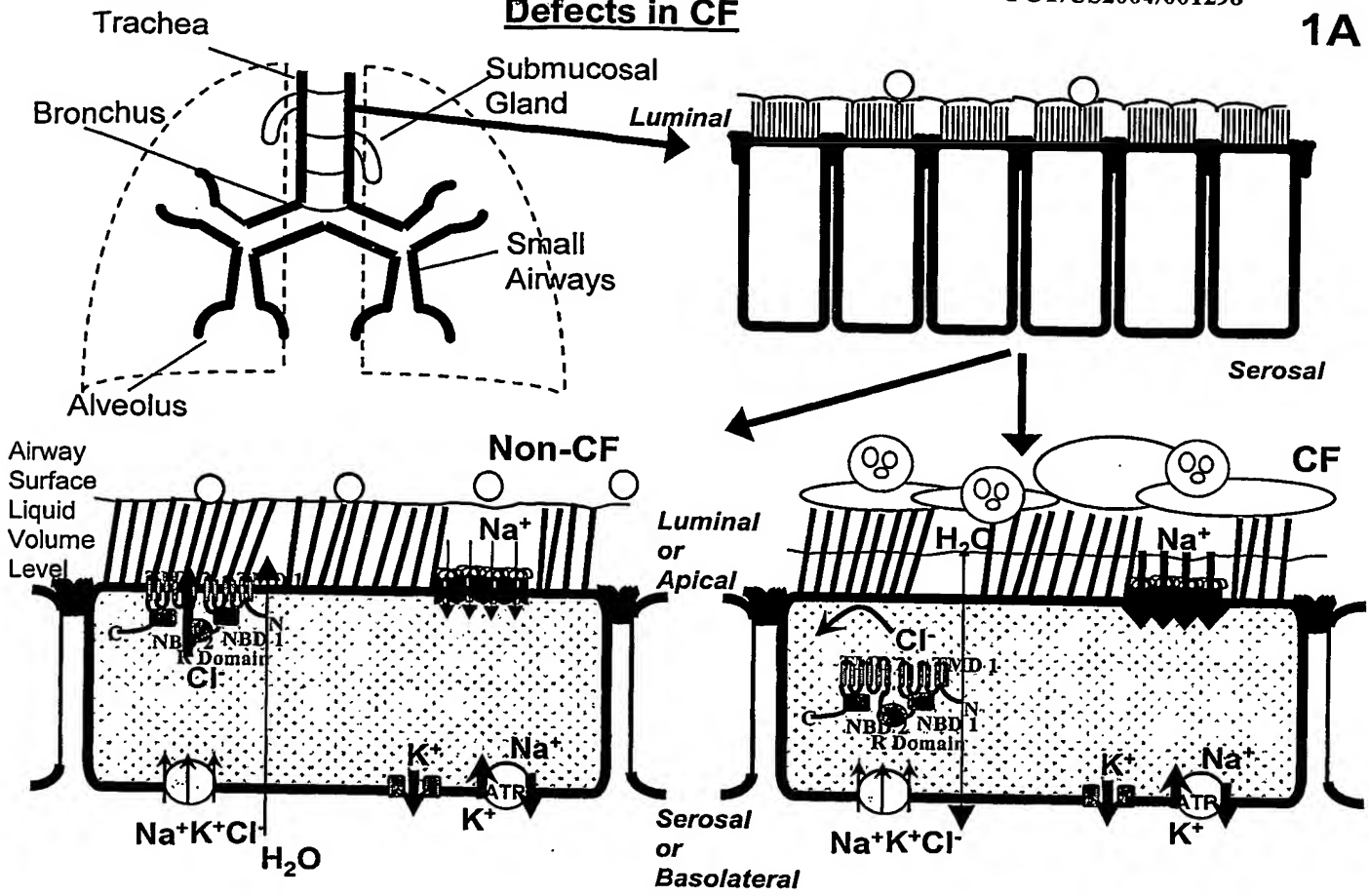
Defects in CF

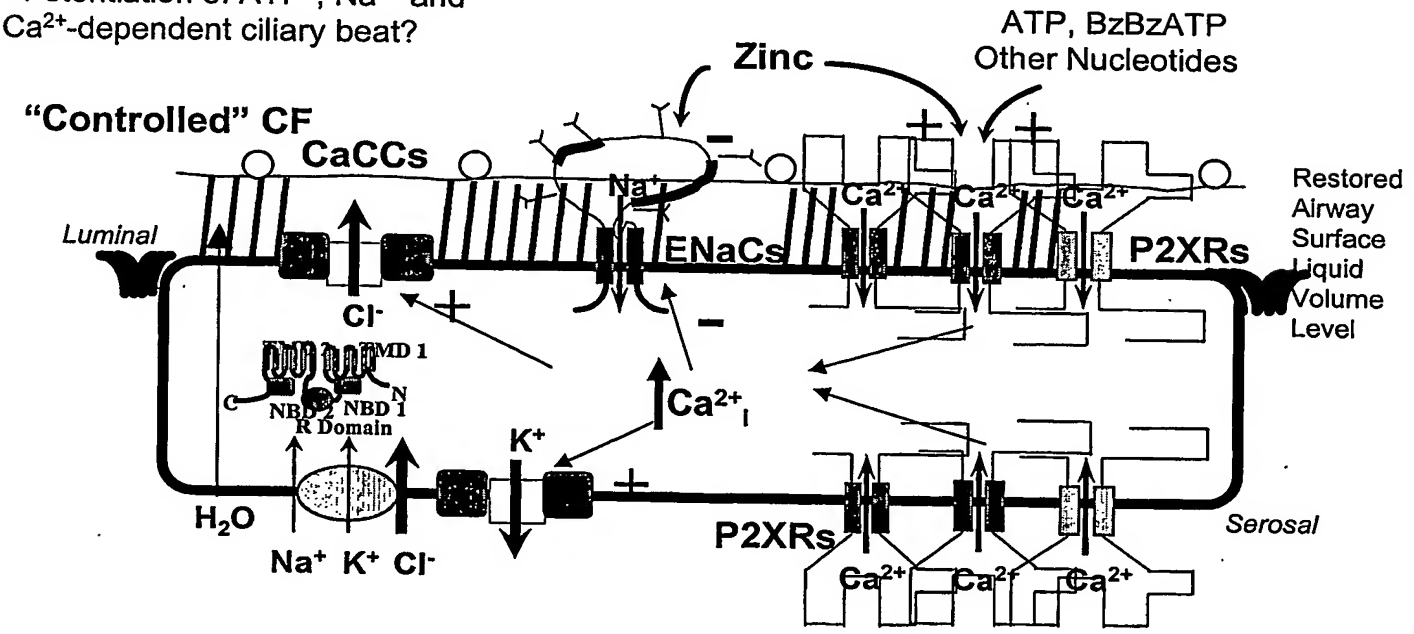
1A



Zinc benefits to CF lung therapy

1B

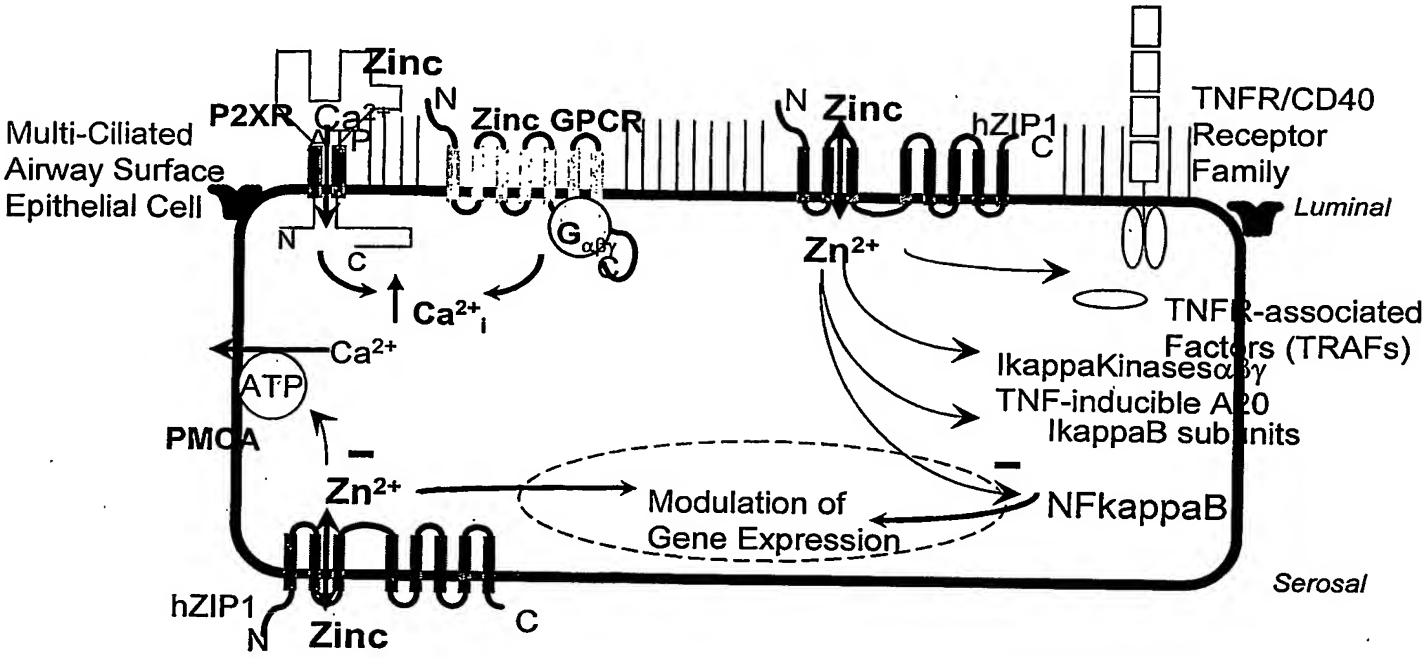
- Rescue of Cl^- and fluid secretion
- Attenuation of Na^+ hyperabsorption
- Potentiation of ATP-, Na^+- and Ca^{2+}-dependent ciliary beat?



Zinc as an anti-inflammatory for CF and other airway diseases such as asthma and common cold

2A

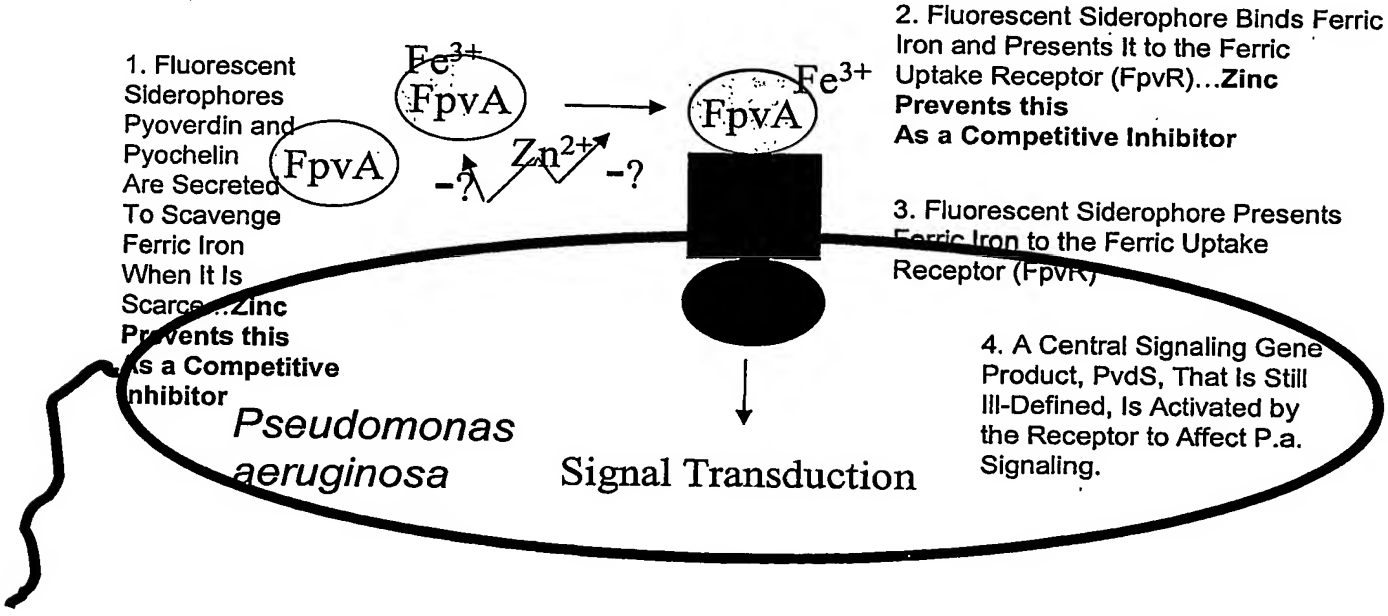
• Zinc in a solution-based formulation enters the cell as free ionic zinc and inhibits NFkappaB activation



Zinc as an anti-microbial for CF and other airway and GI diseases caused by bacterial pathogens

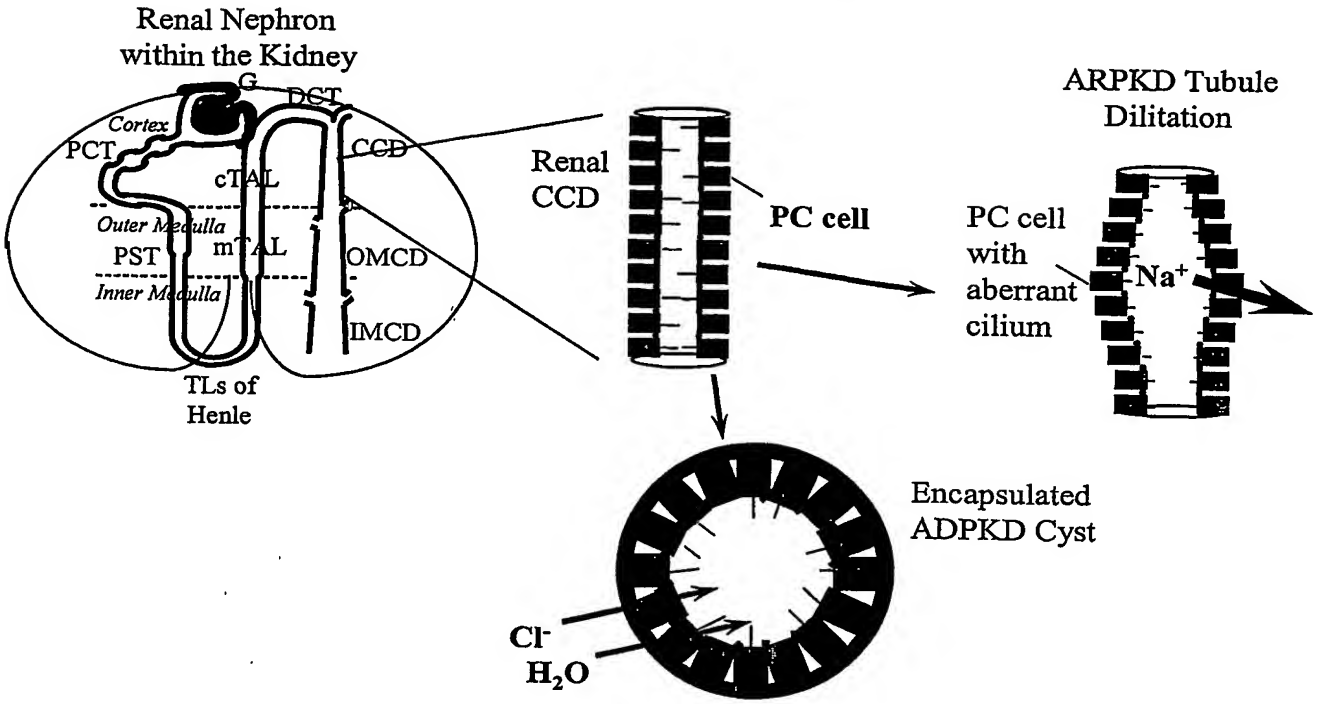
2B

• Zinc in a solution-based formulation competitively inhibits the metal scavenging system of a bacterium.



The two forms of PKD

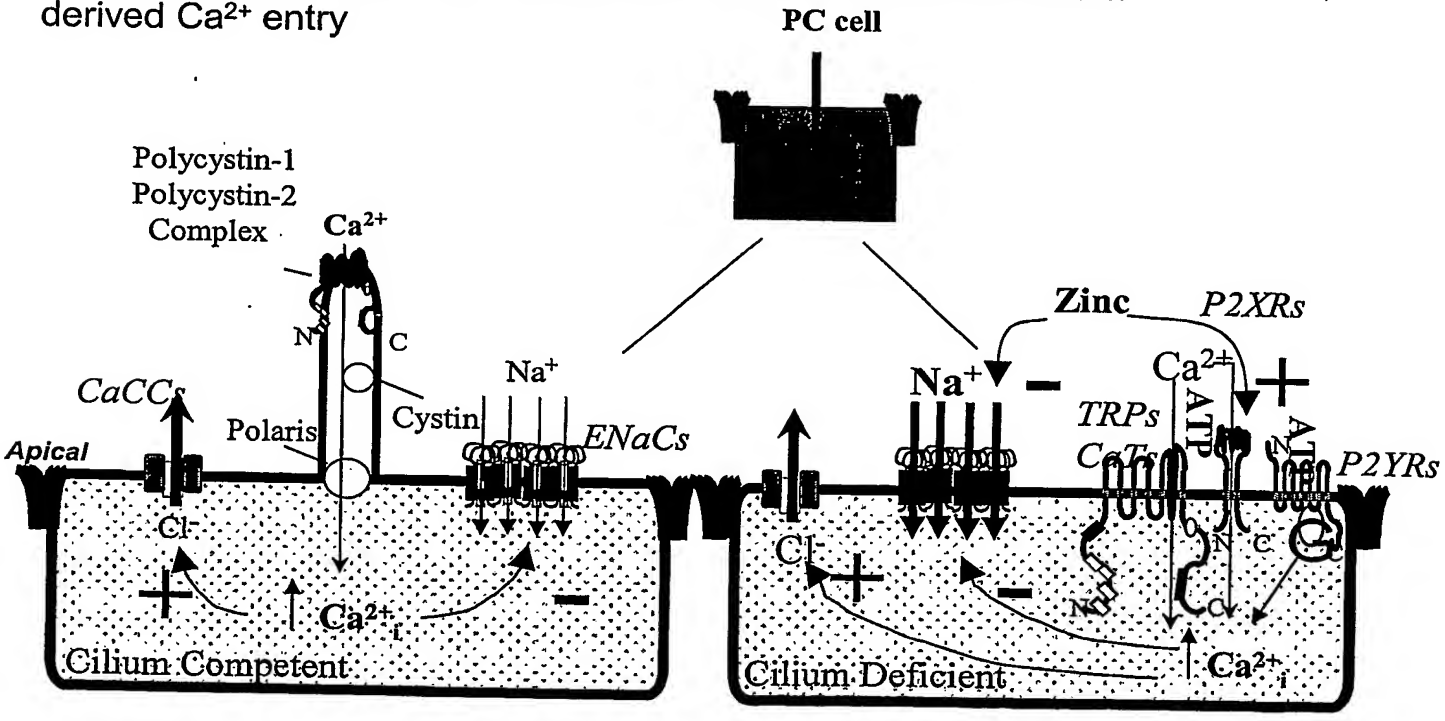
3A



Zinc benefits to PKD therapy and therapy of other renal hypertensive disorders

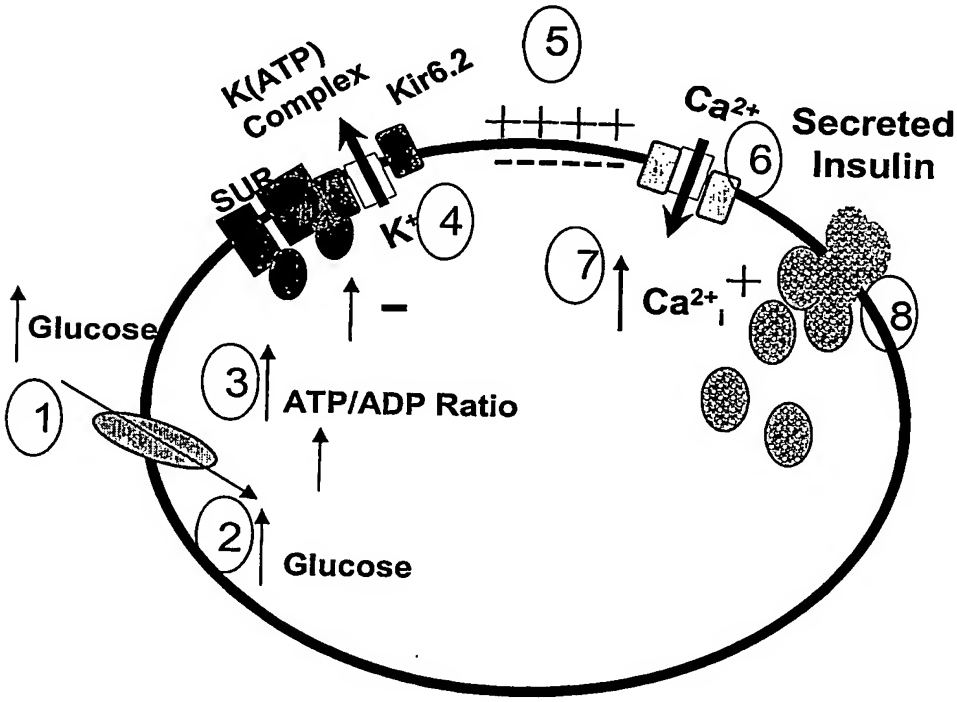
3B

- Direct inhibition of Na^+ hyperabsorption
- Stimulation of P2XR Ca^{2+} entry channels "alternative" to cilium-derived Ca^{2+} entry



Normal Insulin Secretion in a Pancreatic Islet β Cell

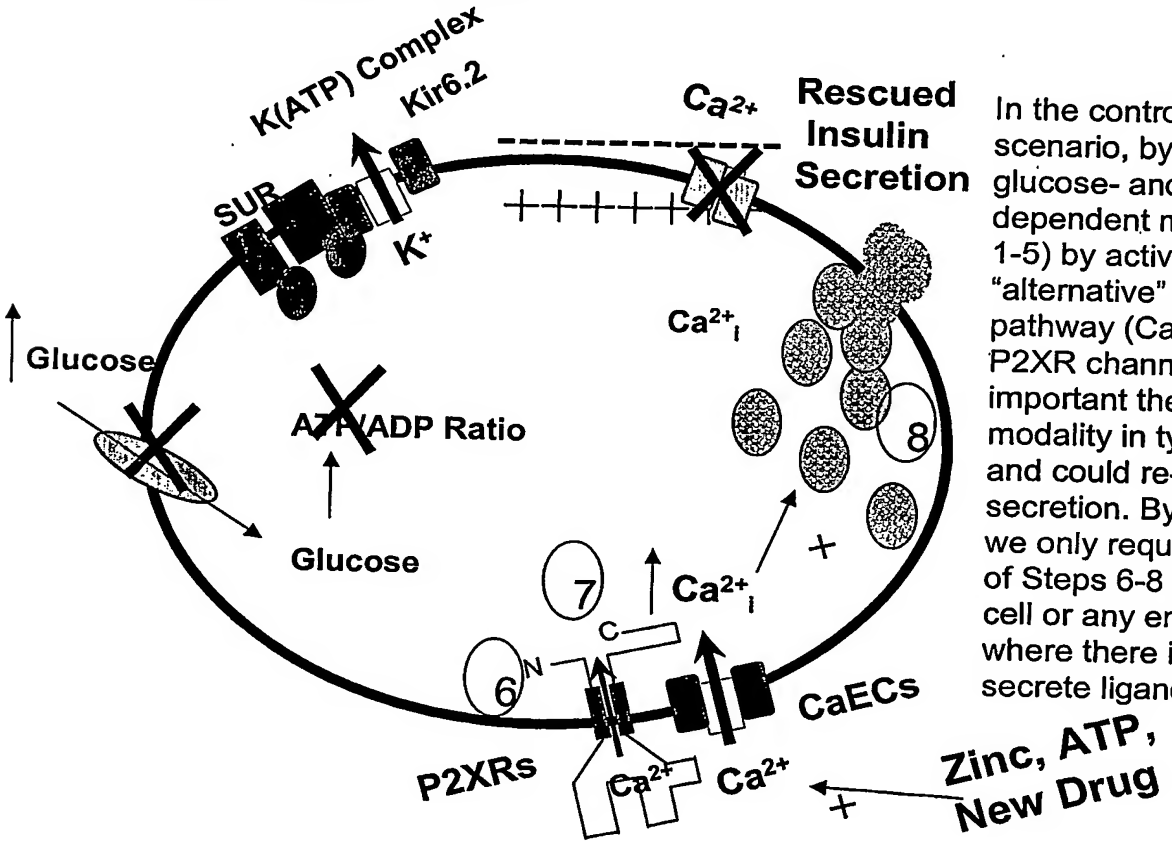
4A



(1) Plasma glucose rises after a meal > (2) glucose enters the cell via GLUT transporters > (3) this causes the cytosolic [ATP] to rise > (4) this inhibits the K(ATP) complex ion channel that is normally basally active to maintain a hyperpolarized membrane potential > (5) closure of this channel depolarizes the β cell membrane > (6) this causes voltage-dependent calcium channels to open > (7) cytosolic calcium rises > (8) the elevation in cell calcium triggers exocytosis of insulin granules.

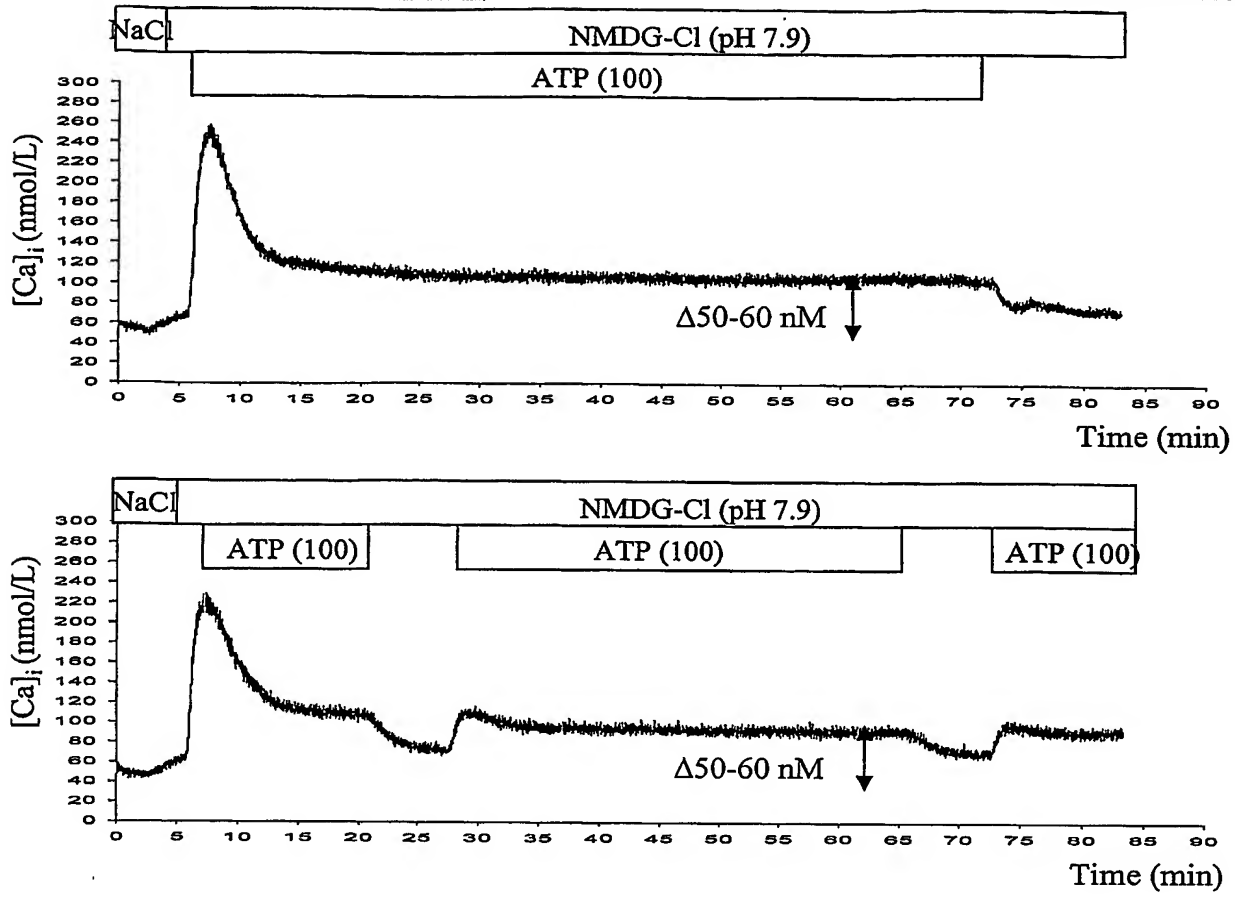
"Controlled" Diabetic β Cell

4B

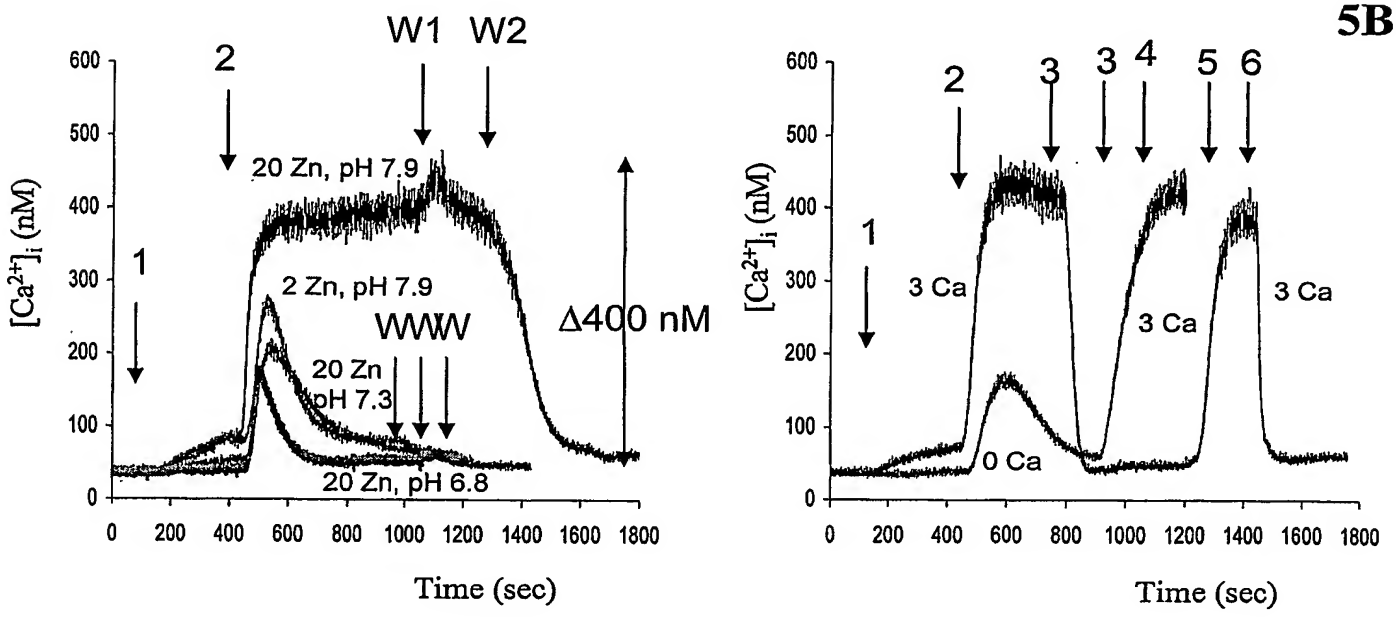


In the controlled diabetic scenario, by-passing the glucose- and voltage-dependent mechanism (Steps 1-5) by activating an "alternative" calcium entry pathway (CaEC), like the P2XR channels, could be an important therapeutic modality in type II diabetes and could re-stimulate insulin secretion. By this approach, we only require re-capitulation of Steps 6-8 for the diabetic β cell or any endocrine cell where there is failure to secrete ligand.

5A



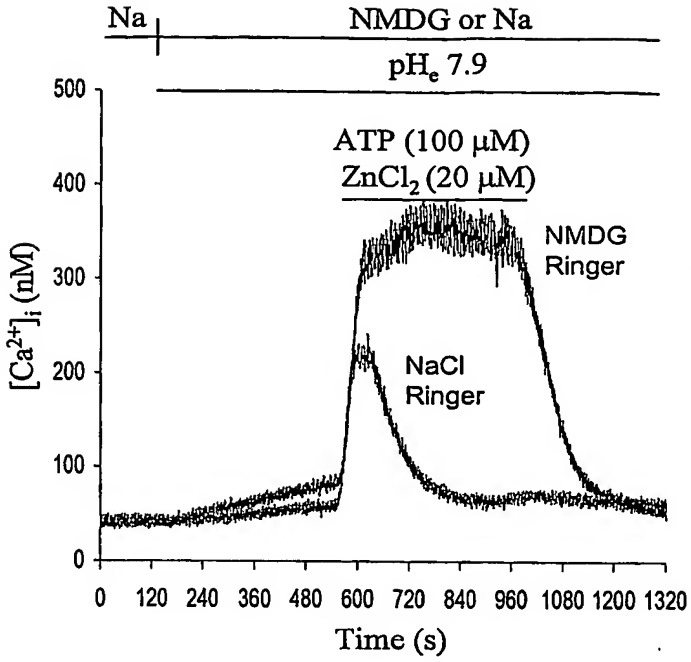
5B



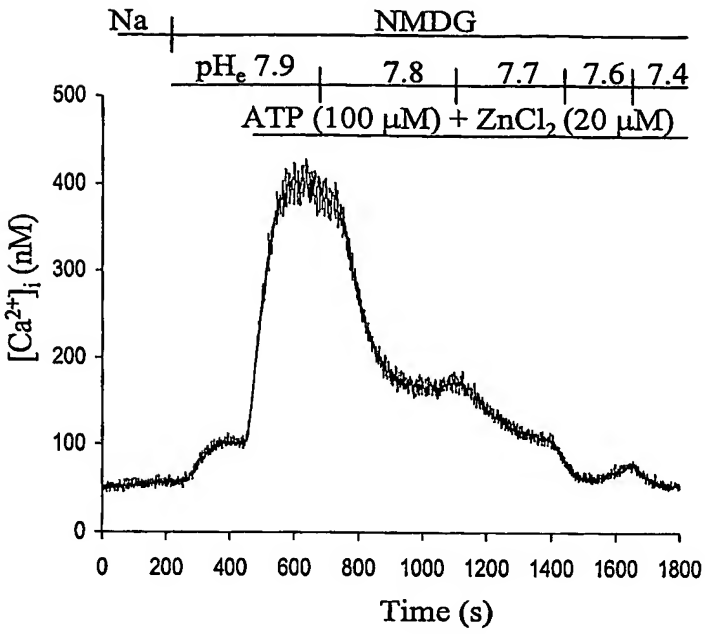
Black = 100 ATP, 20 Zn, pH 7.9
Red = 100 ATP, 2 Zn, pH 7.9
Blue = 100 ATP, 20 Zn, pH 7.3
Green = 100 ATP, 20 Zn, pH 6.8

Black = 20 Zn, pH 7.9 plus Extracellular Ca^{2+}
Red = 20 Zn, pH 7.9, 0 Extracellular Ca^{2+}, then, add back 3 mM Ca^{2+}

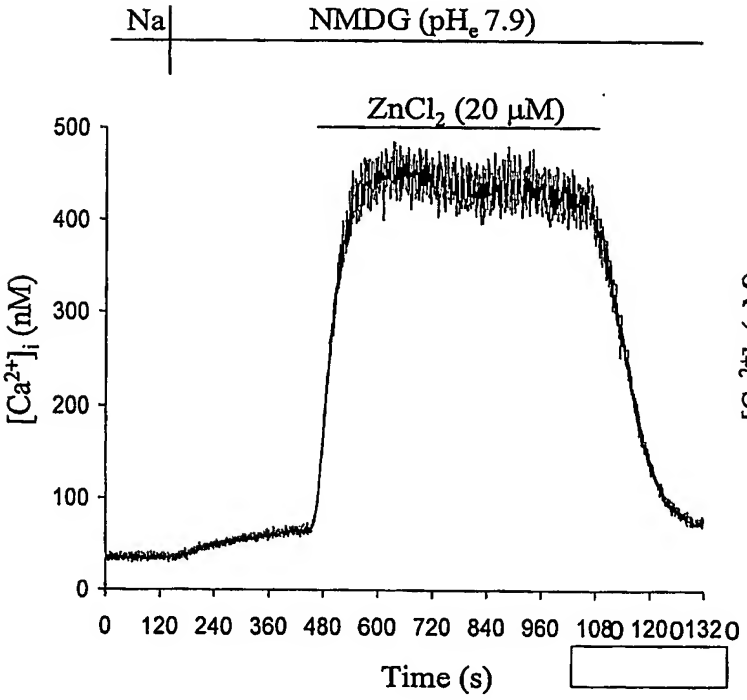
5C


Na
NMDG or Na
pH$_e$ 7.9
ATP (100 μM)
ZnCl$_2$ (20 μM)
NMDG Ringer
NaCl Ringer
[Ca^{2+}]$_i$ (nM)
Time (s)

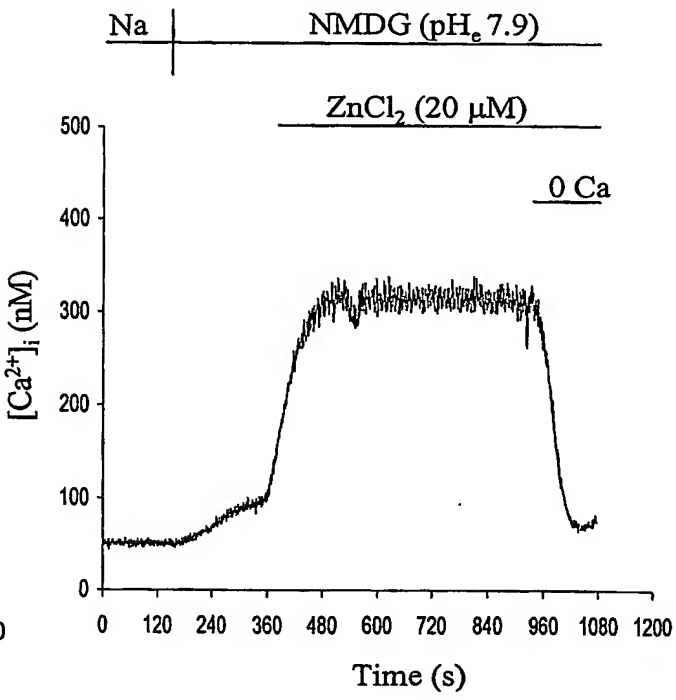
5D


Na
NMDG
pH$_e$ 7.9
7.8
7.7
7.6
7.4
ATP (100 μM) + ZnCl$_2$ (20 μM)
[Ca^{2+}]$_i$ (nM)
Time (s)

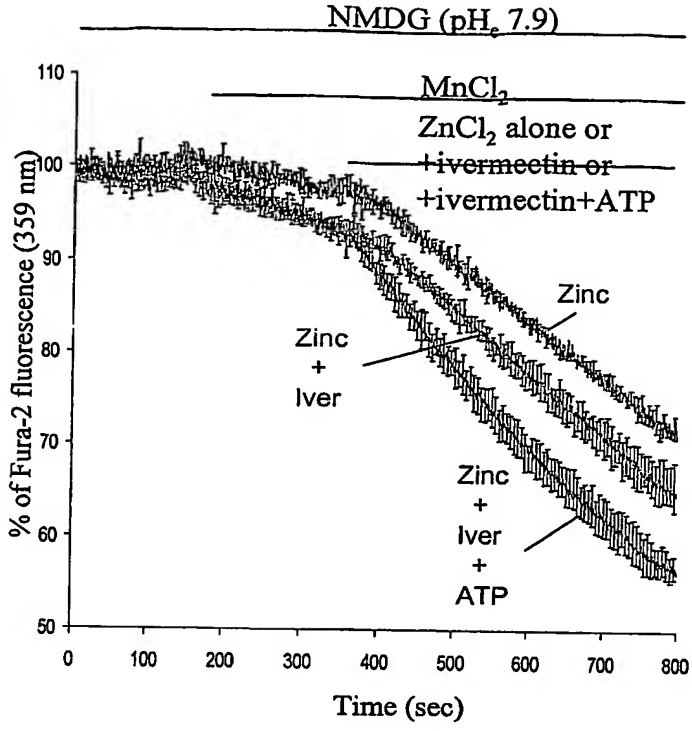
5E

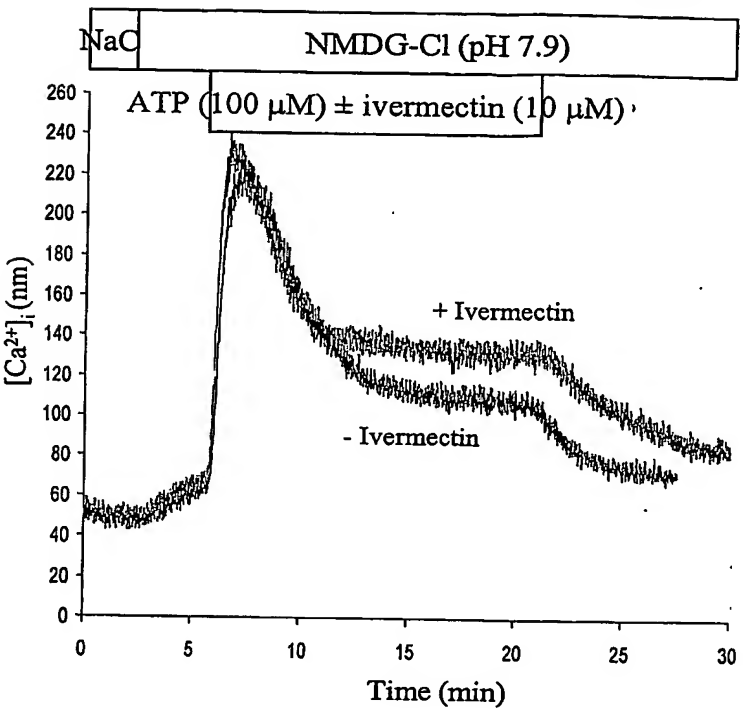

Na
NMDG (pH$_e$ 7.9)
ZnCl$_2$ (20 μM)
[Ca^{2+}]$_i$ (nM)
Time (s)

5F

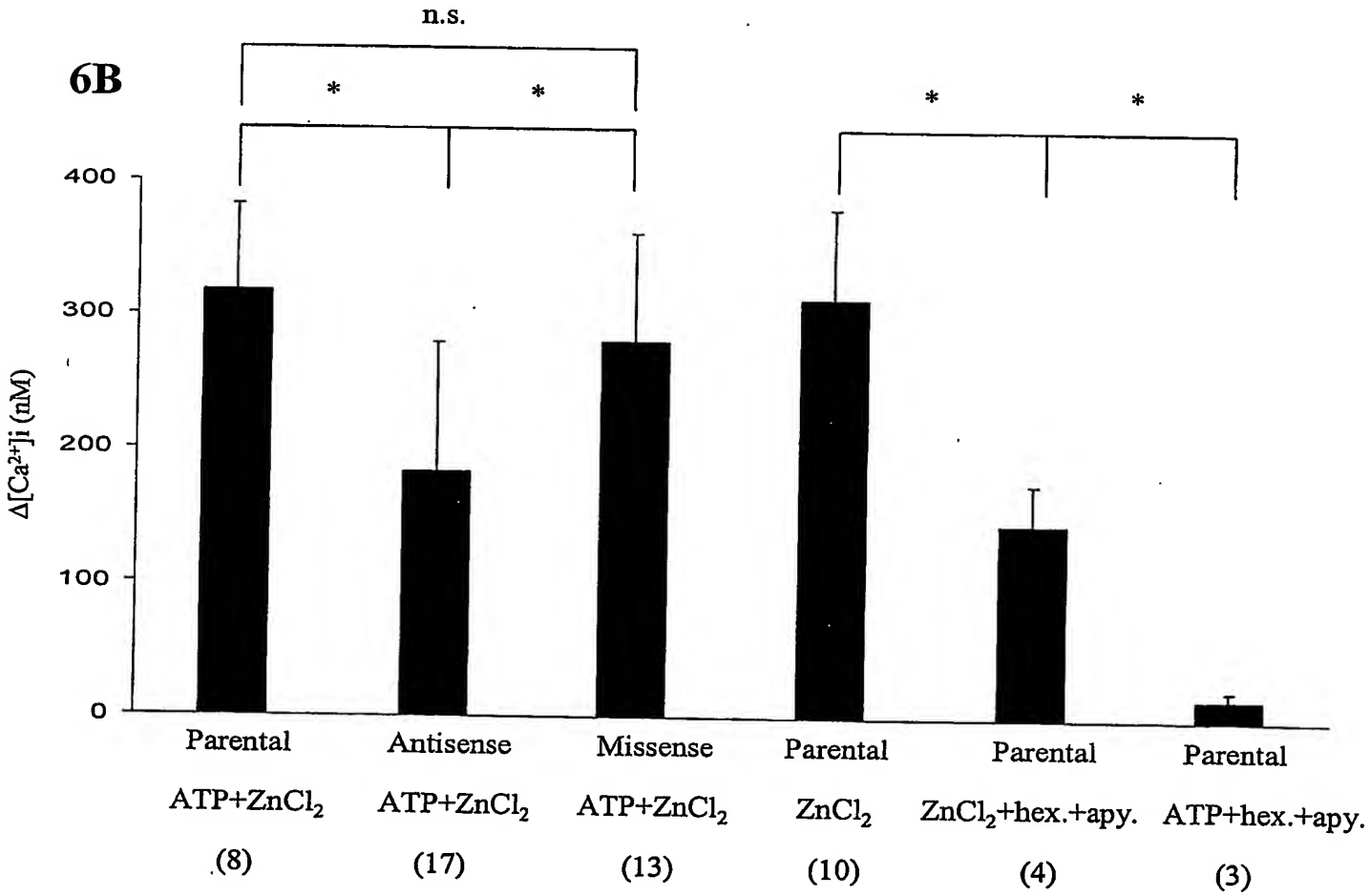

Na
NMDG (pH$_e$ 7.9)
ZnCl$_2$ (20 μM)
0 Ca
[Ca^{2+}]$_i$ (nM)
Time (s)

6A

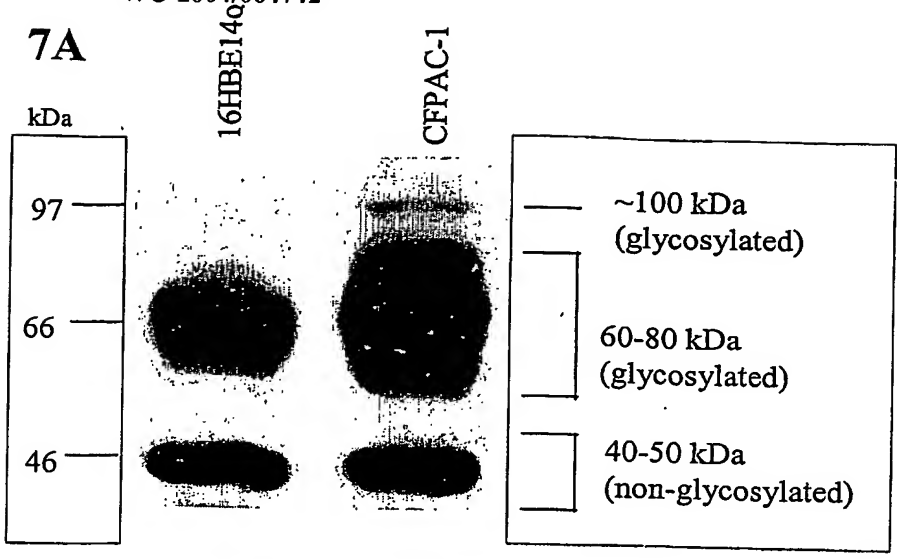

NMDG (pH$_e$ 7.9)
MnCl$_2$
ZnCl$_2$ alone or
+ivermectin or
+ivermectin+ATP
Zinc
Zinc + Iver
Zinc + Iver + ATP
% of Fura-2 fluorescence (359 nm)
Time (sec)


NaC
NMDG-Cl (pH 7.9)
ATP (100 μM) ± ivermectin (10 μM)
+ Ivermectin
- Ivermectin
[Ca2+]i (nm)
Time (min)

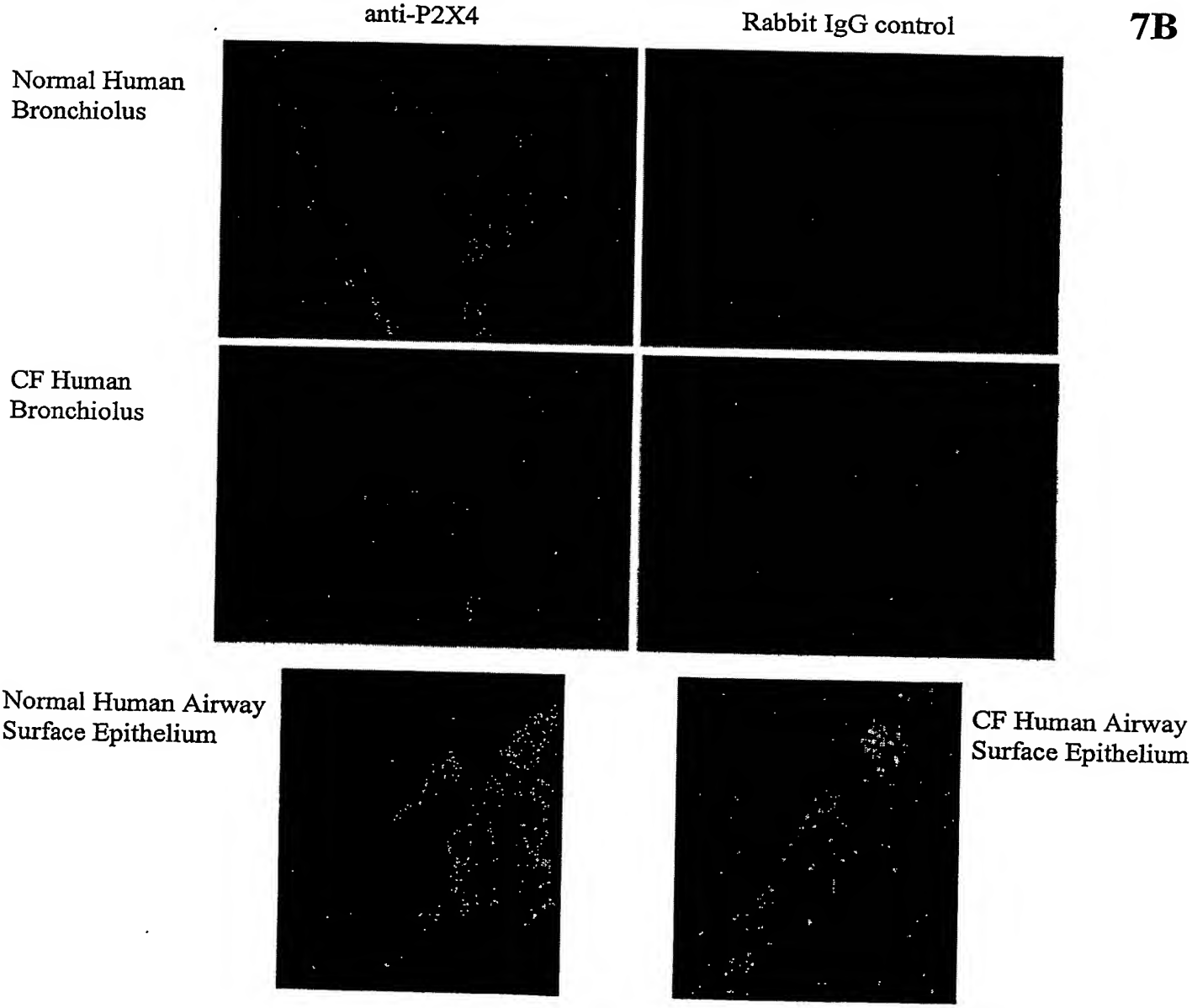
6B


n.s.
*
*
*
*
Δ[Ca2+]i (nM)
Parental ATP+ZnCl2 (8)
Antisense ATP+ZnCl2 (17)
Missense ATP+ZnCl2 (13)
Parental ZnCl2 (10)
Parental ZnCl2+hex.+apy. (4)
Parental ATP+hex.+apy. (3)

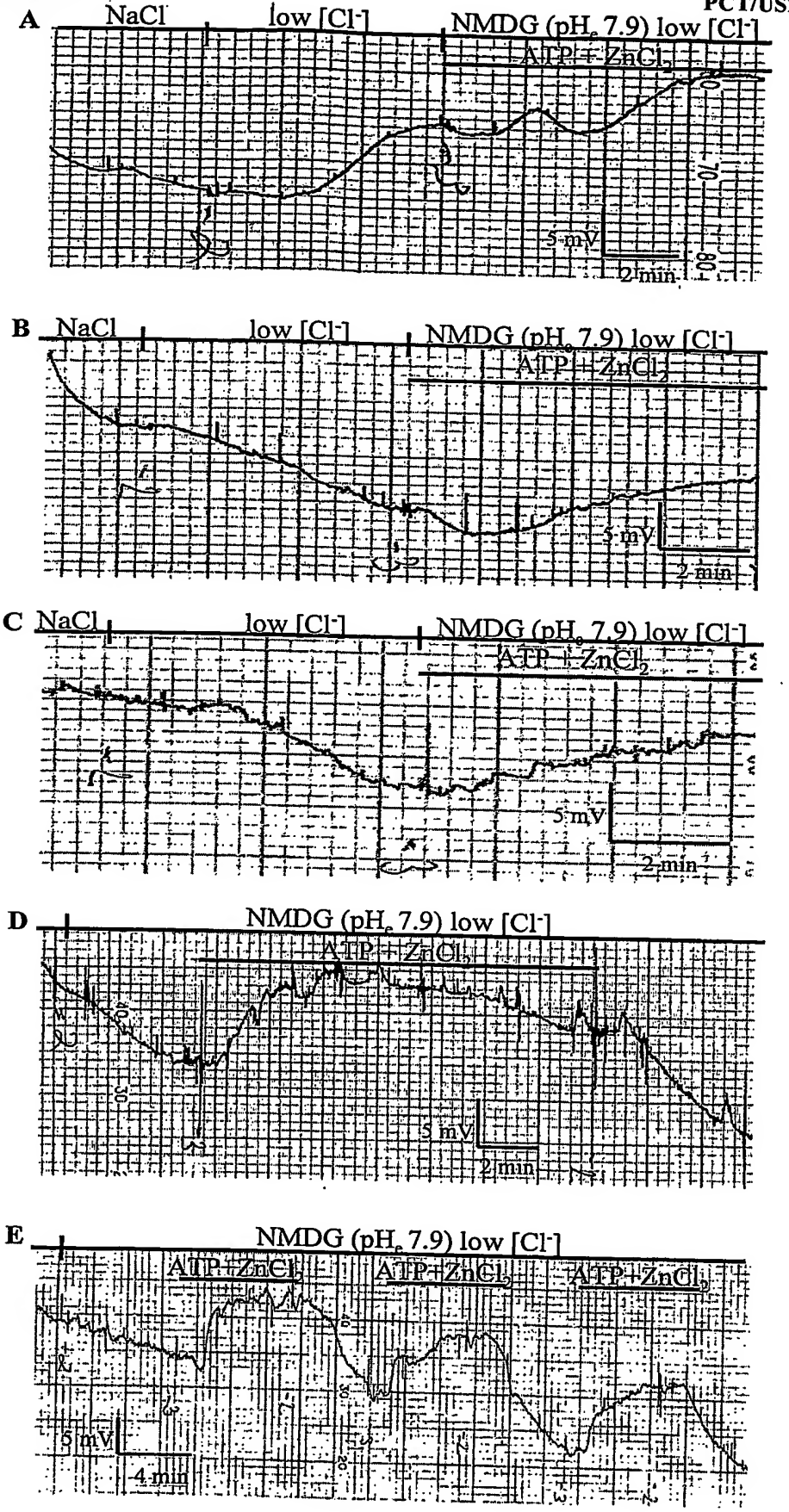
7A


kDa
16HBE14o
CFPAC-1
97
66
46
~100 kDa
(glycosylated)
60-80 kDa
(glycosylated)
40-50 kDa
(non-glycosylated)

7B


anti-P2X4
Rabbit IgG control
Normal Human Bronchiolus
CF Human Bronchiolus
Normal Human Airway Surface Epithelium
CF Human Airway Surface Epithelium

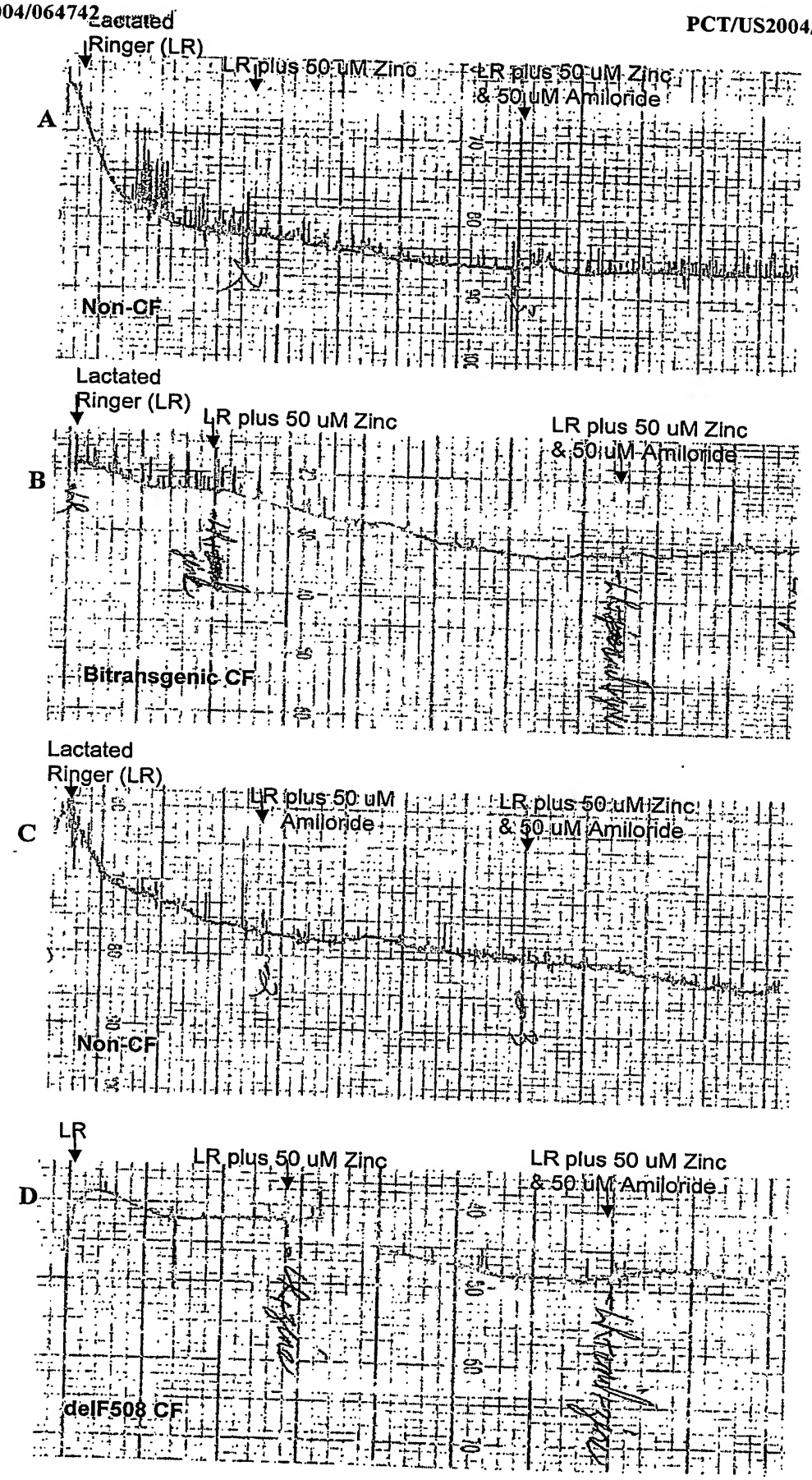
8A-E

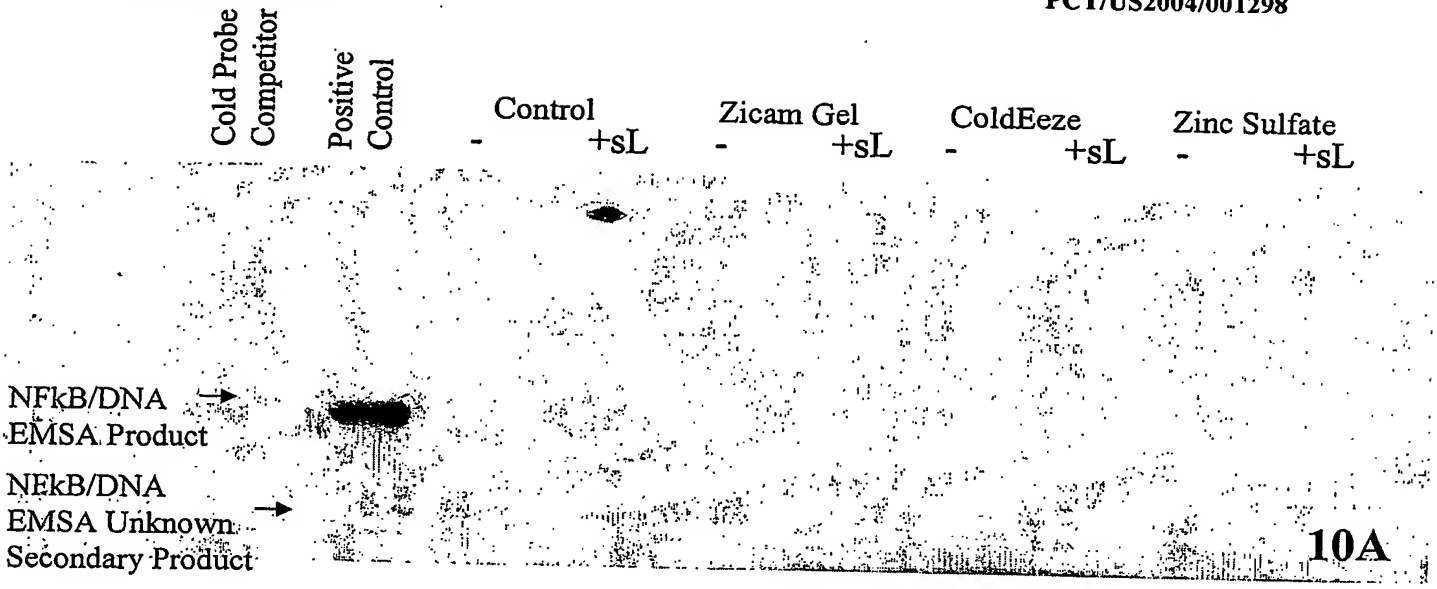

A
NaCl
low [Cl-]
NMDG (pHo 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
B
NaCl
low [Cl-]
NMDG (pHo 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
C
NaCl
low [Cl-]
NMDG (pHo 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
D
NMDG (pHo 7.9) low [Cl-]
ATP + ZnCl2
5 mV
2 min
E
NMDG (pHo 7.9) low [Cl-]
ATP+ZnCl2
ATP+ZnCl2
ATP+ZnCl2
5 mV
4 min

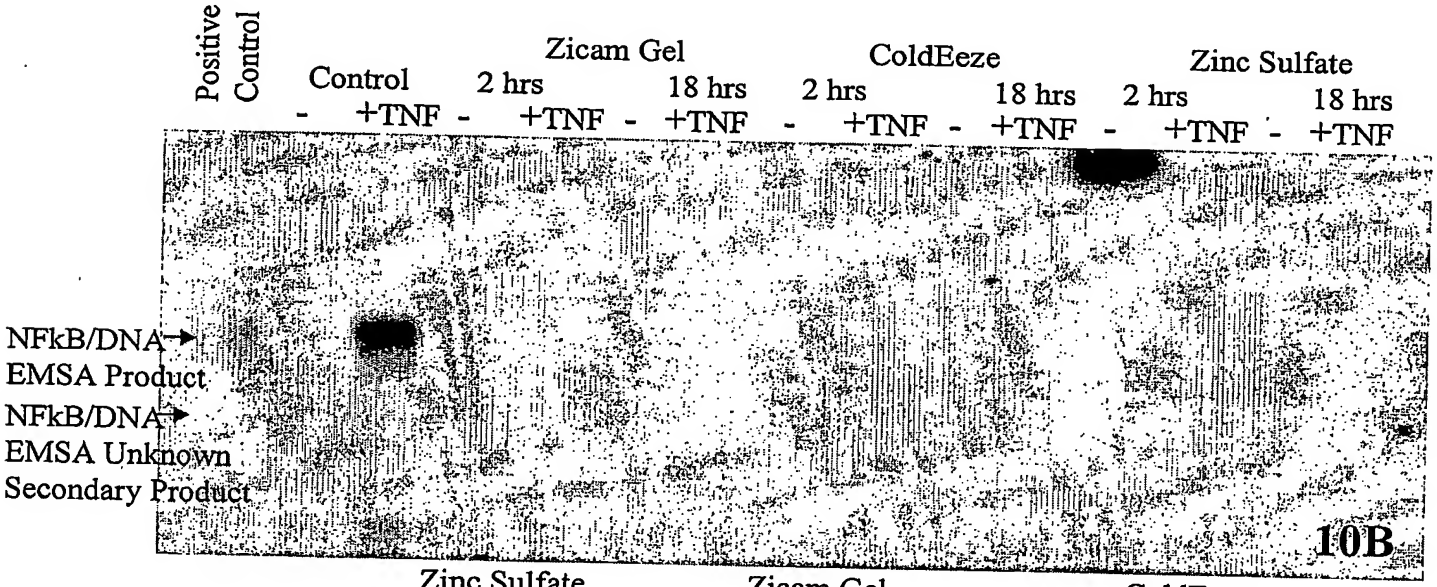
8F

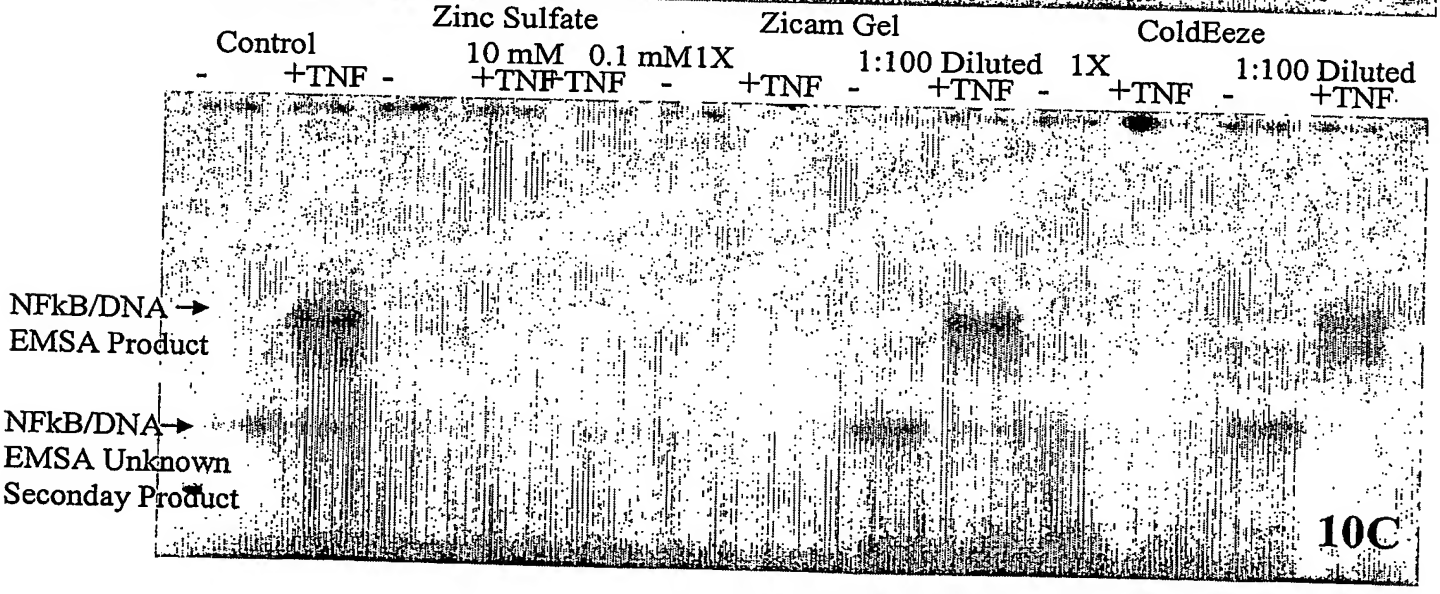
Transepithelial Nasal Potential Difference Values of Control, Δ508 CF and Bitransgenic CF Mice

	Control Cftr(+/-)	n	CF Cftr(ΔF508/ΔF508)	n	Bitransgenic CF Cftr(-/-)	n
Starting point	-18.7 ± 6.5	19	-26.3 ± 7.2*	11	-26.1 ± 3.8*	14
Low $[Cl^-]_e$ (Na^+; pH:7.3)	-5.5 ± 1.5	8	+3.7 ± 1.6*	3	+4.8 ± 2.5*	7
ATP + $ZnCl_2$ (NMDG; pH:7.9)	-4.7 ± 1.8	6	-4.0 ± 2.0	3	-3.8 ± 2.0	12
Low $[Cl^-]_e$ (Na^+; pH:7.9)	-4.8 ± 2.0	6	+5.4 ± 2.8*	7	+6.7 ± 4.0*	3
ATP + $ZnCl_2$ (NMDG; pH:7.9)	-6.0 ± 1.4	2	-9.4 ± 1.6*#	8	-9.7 ± 3.1*&	3
Low $[Cl^-]_e$ (NMDG; pH:7.9)	-4.8 ± 3.3	5			+5.8 ± 1.9*	4
ATP + $ZnCl_2$ (NMDG; pH:7.9)	-5.7 ± 1.2	3			-10.2 ± 1.3*&	6
ATP alone (NMDG; pH:7.9)					-2.3 ± 1.0§	4
Low $[Cl^-]_e$ (NMDG; no added Ca^{2+}; pH:7.9)	-7.3 ± 0.6	3			+6.0 ± 0.8*	4
ATP + $ZnCl_2$ (NMDG; no added Ca^{2+}; pH:7.9)	-1.3 ± 0.6$	3			-2.0 ± 1.2$	4


A
Lactated Ringer (LR)
LR plus 50 uM Zinc
LR plus 50 uM Zinc & 50 uM Amiloride
Non-CF
B
Lactated Ringer (LR)
LR plus 50 uM Zinc
LR plus 50 uM Zinc & 50 uM Amiloride
Bitransgenic CF
C
Lactated Ringer (LR)
LR plus 50 uM Amiloride
LR plus 50 uM Zinc & 50 uM Amiloride
Non-CF
D
LR
LR plus 50 uM Zinc
LR plus 50 uM Zinc & 50 uM Amiloride
delF508 CF


Cold Probe Competitor
Positive Control
Control
Zicam Gel
ColdEeze
Zinc Sulfate
- +sL - +sL - +sL - +sL
NFkB/DNA EMSA Product
NEkB/DNA EMSA Unknown Secondary Product
10A

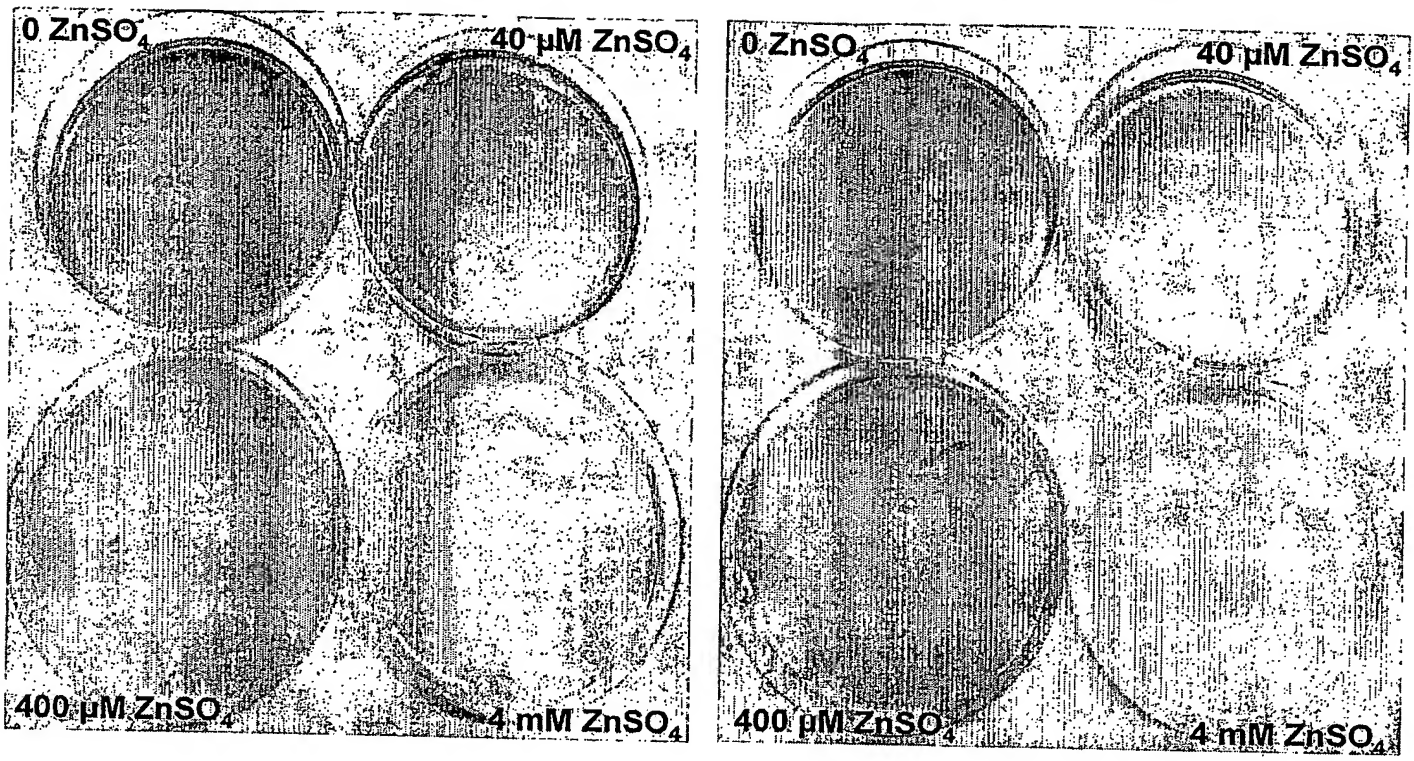

Positive Control
Control
Zicam Gel
ColdEeze
Zinc Sulfate
2 hrs 18 hrs 2 hrs 18 hrs 2 hrs 18 hrs
- +TNF - +TNF - +TNF - +TNF - +TNF - +TNF - +TNF
NFkB/DNA EMSA Product
NFkB/DNA EMSA Unknown Secondary Product
10B


Control
Zinc Sulfate
Zicam Gel
ColdEeze
10 mM 0.1 mM 1X 1:100 Diluted 1X 1:100 Diluted
- +TNF - +TNF +TNF - +TNF - +TNF - +TNF - +TNF
NFkB/DNA EMSA Product
NFkB/DNA EMSA Unknown Seconday Product
10C

Non-mucoid *P.a.*

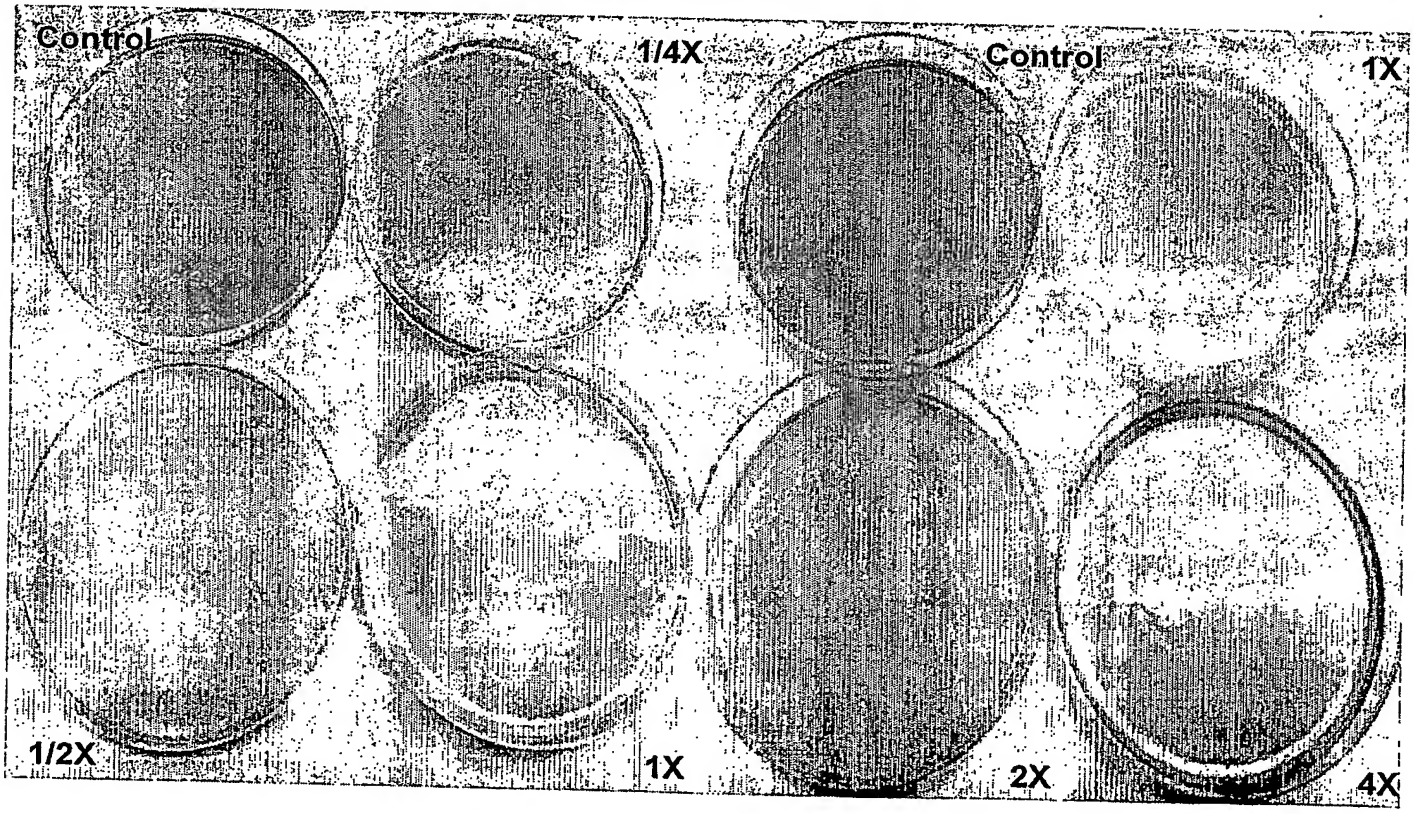
Mucoid *P.a.*

11A

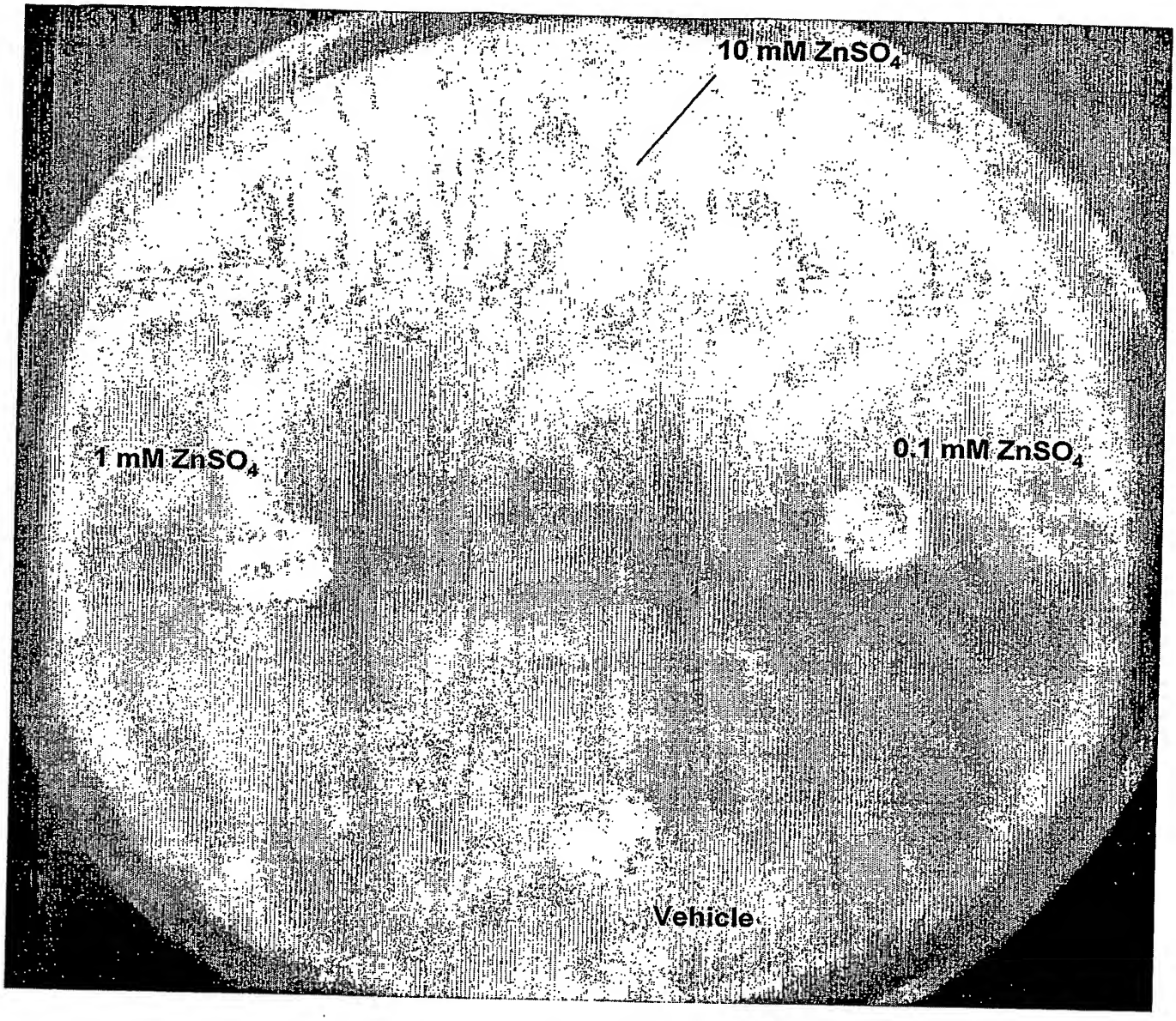


Mucoid *P.a.*

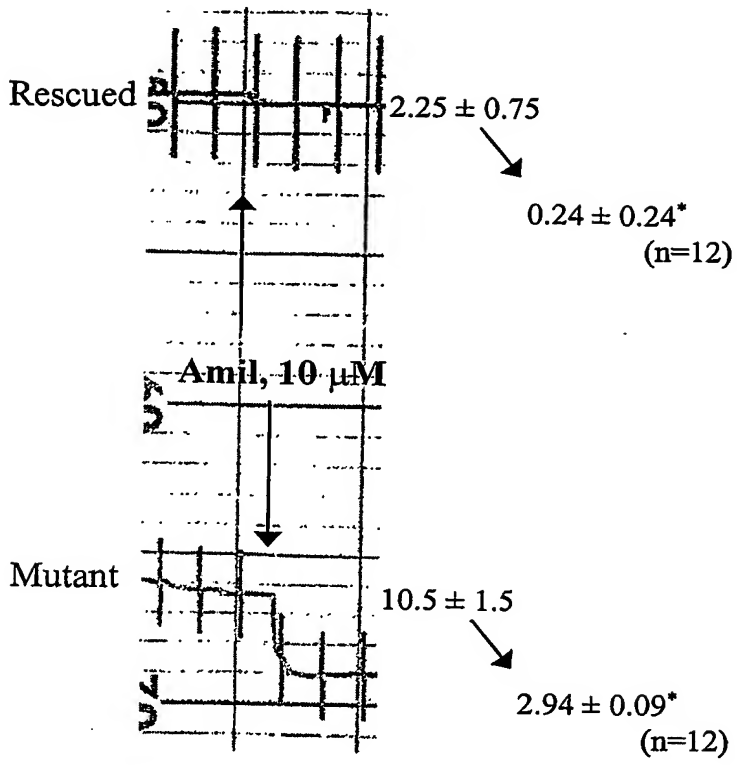
11B



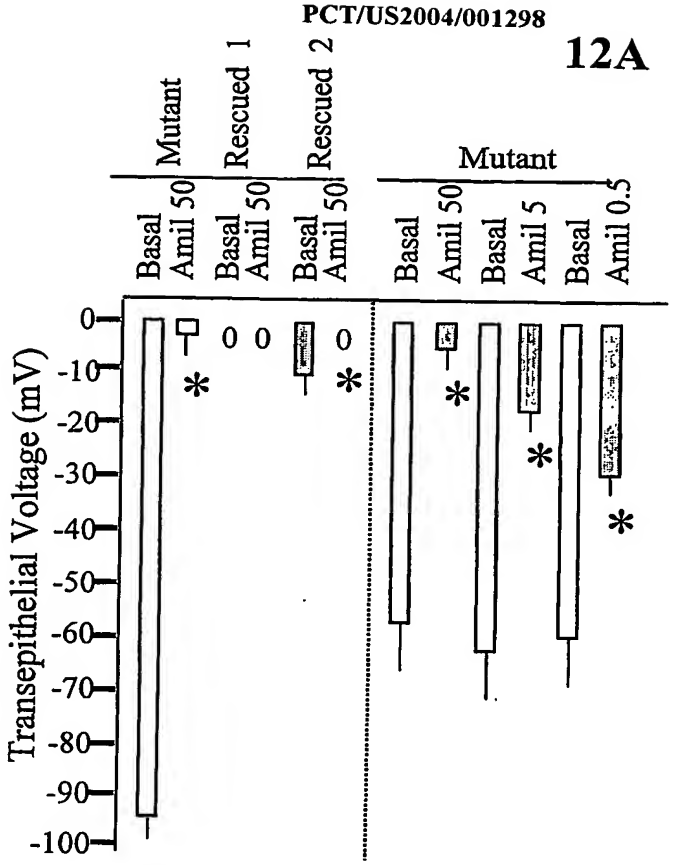
11C

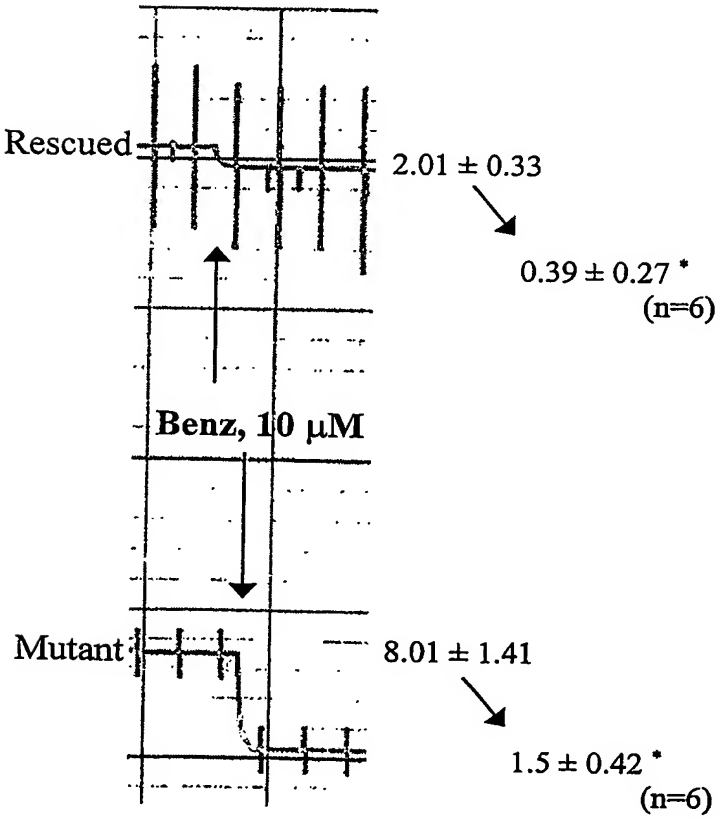


E. coli.

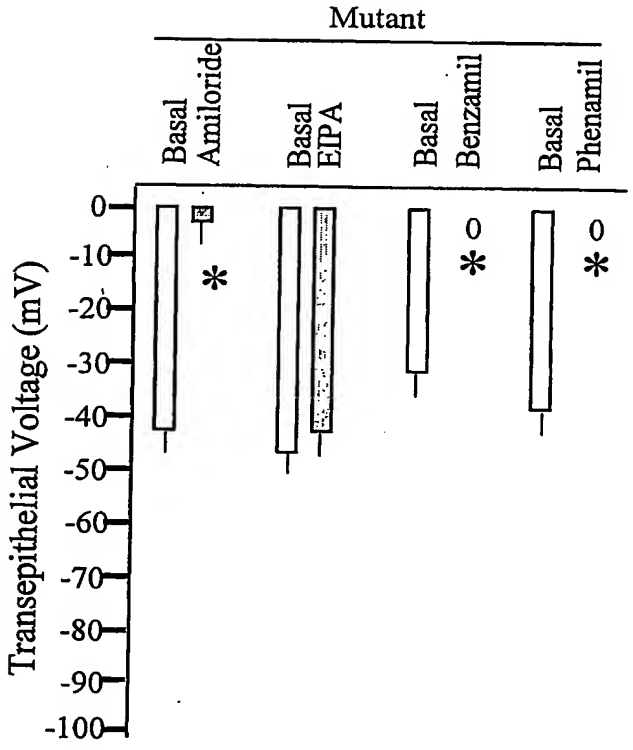

Rescued
2.25 ± 0.75
0.24 ± 0.24*
(n=12)
Amil, 10 μM
Mutant
10.5 ± 1.5
2.94 ± 0.09*
(n=12)

12A

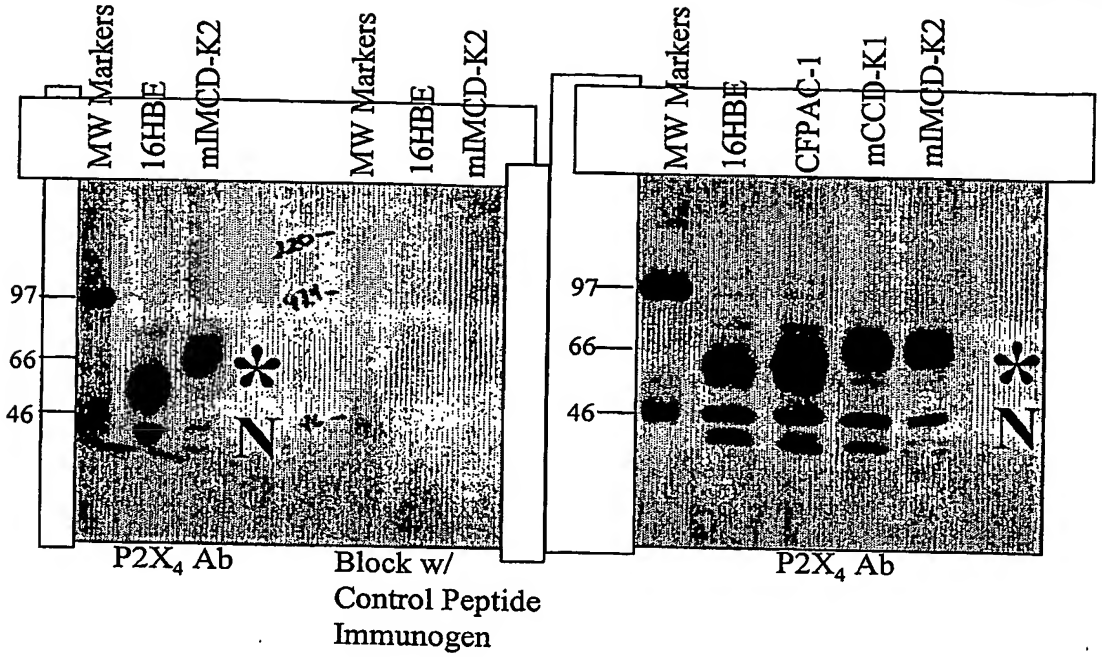

Mutant
Rescued 1
Rescued 2
Mutant
Basal
Amil 50
Basal
Amil 50
Basal
Amil 50
Basal
Amil 50
Basal
Amil 5
Basal
Amil 0.5
0 0
0
Transepithelial Voltage (mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100


Rescued
2.01 ± 0.33
0.39 ± 0.27 *
(n=6)
Benz, 10 μM
Mutant
8.01 ± 1.41
1.5 ± 0.42 *
(n=6)

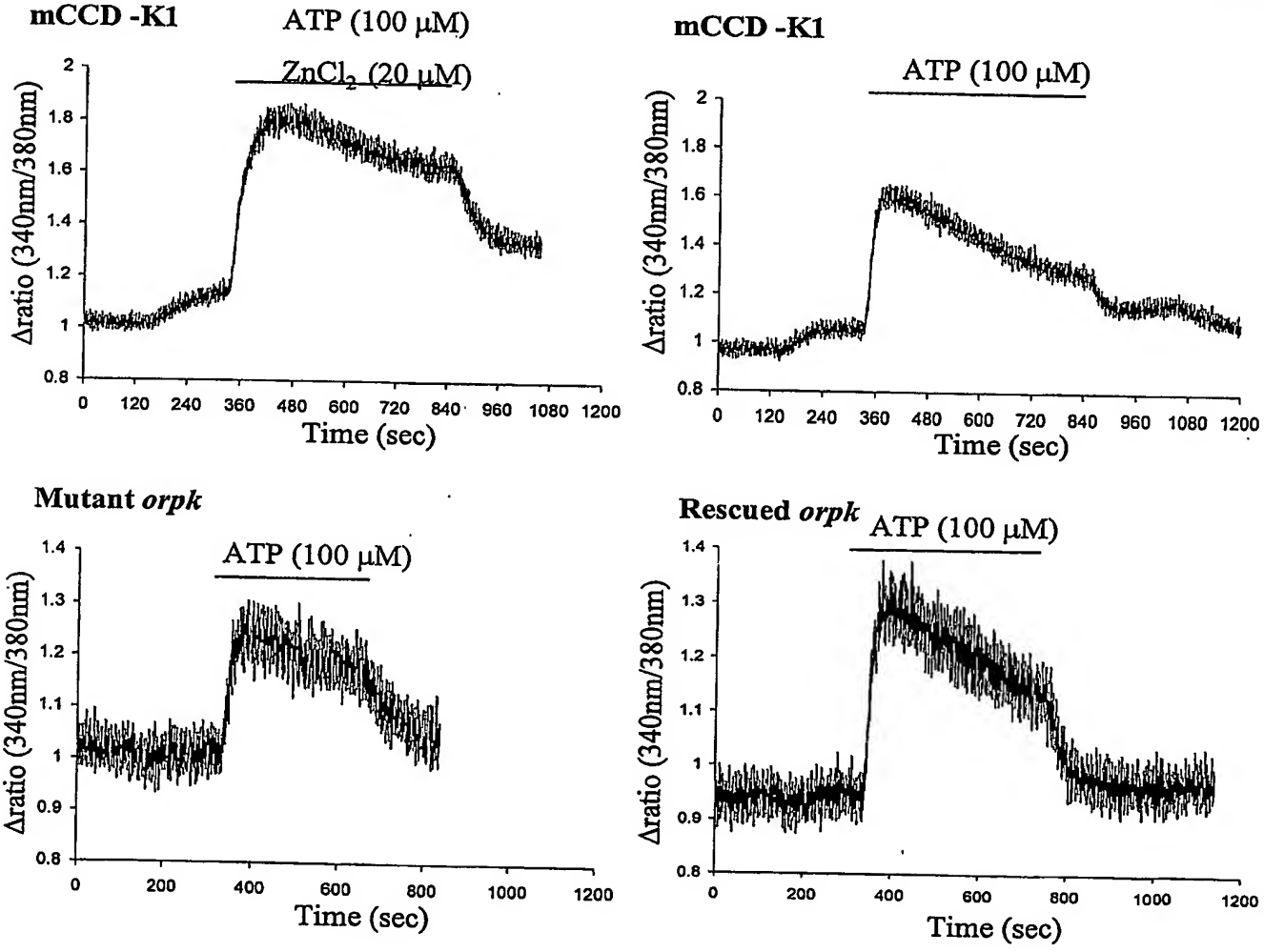
12B


Mutant
Basal
Amiloride
Basal
EIPA
Basal
Benzamil
Basal
Phenamil
0
0
Transepithelial Voltage (mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100

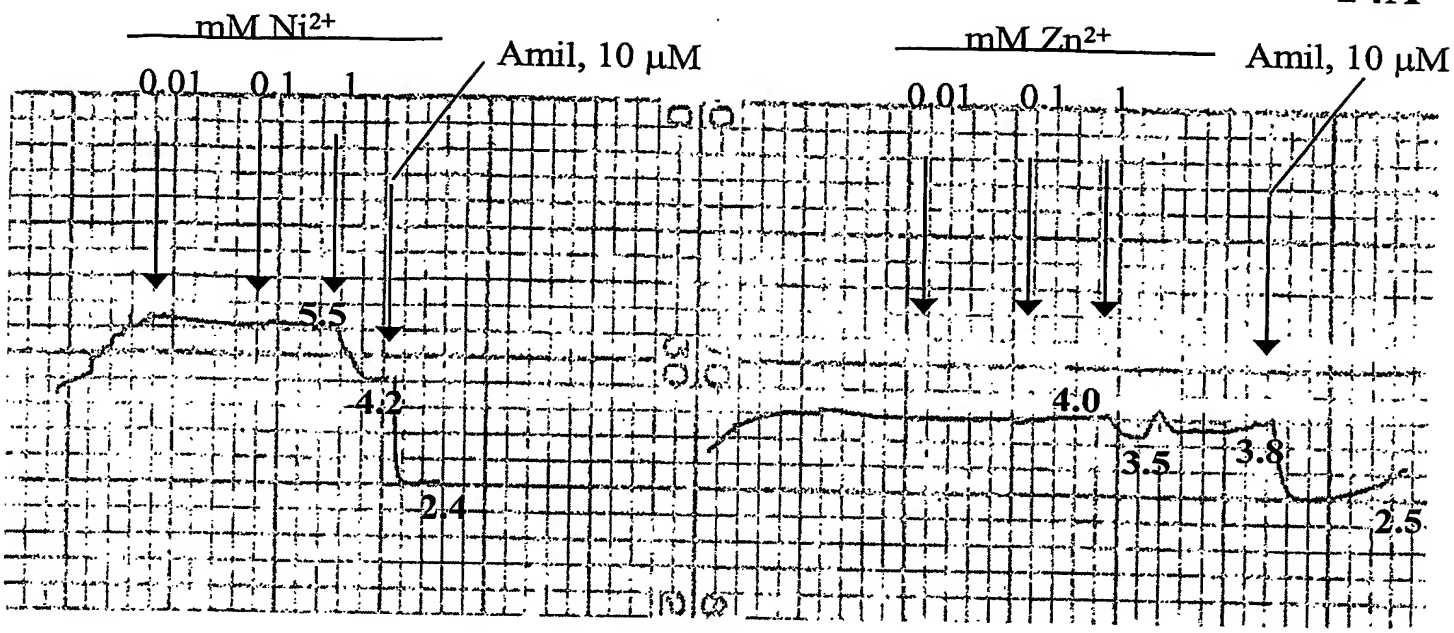
13A


MW Markers
16HBE
mIMCD-K2
MW Markers
16HBE
mIMCD-K2
97
66
46
N
P2X4 Ab
Block w/
Control Peptide
Immunogen
MW Markers
16HBE
CFPAC-1
mCCD-K1
mIMCD-K2
97
66
46
N
P2X4 Ab

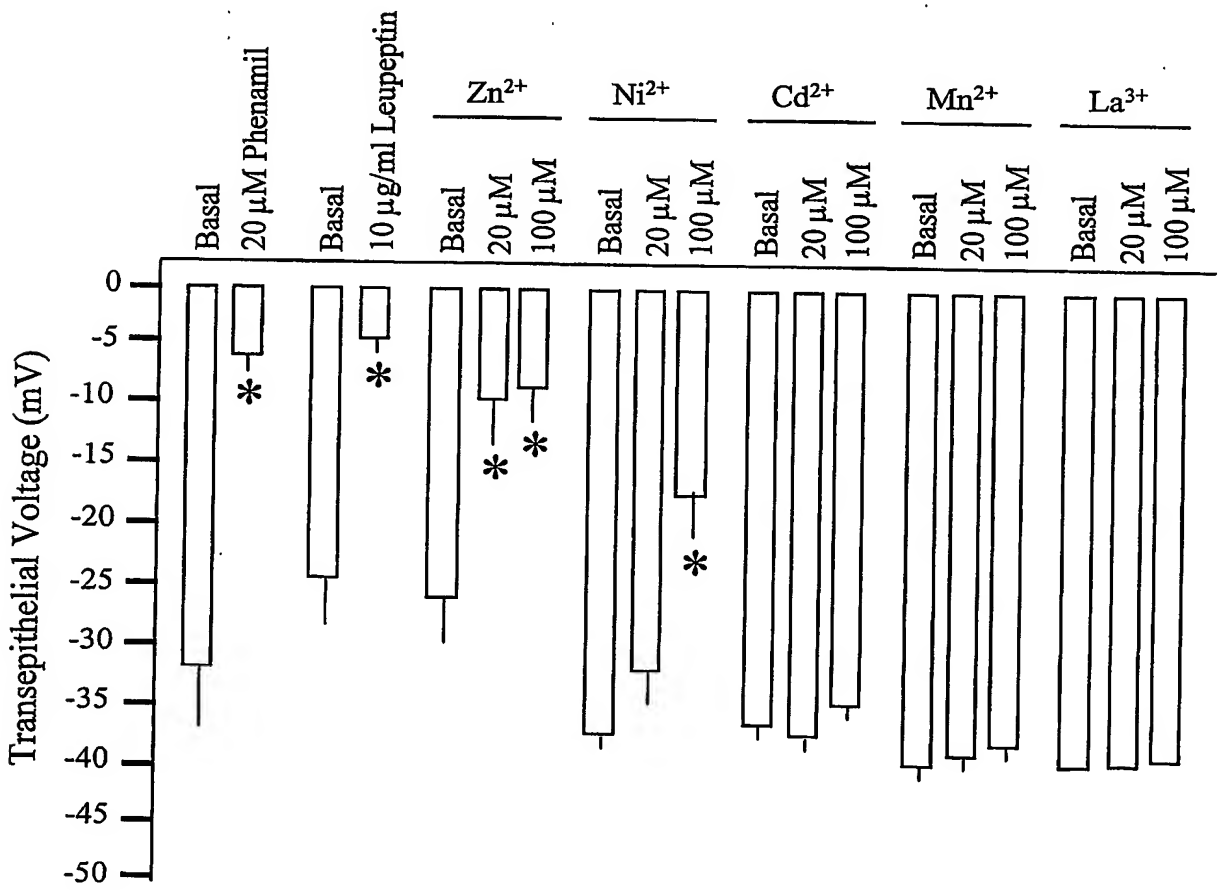
13B


mCCD -K1
ATP (100 μM)
ZnCl2 (20 μM)
Δratio (340nm/380nm)
Time (sec)
mCCD -K1
ATP (100 μM)
Δratio (340nm/380nm)
Time (sec)
Mutant orpk
ATP (100 μM)
Δratio (340nm/380nm)
Time (sec)
Rescued orpk
ATP (100 μM)
Δratio (340nm/380nm)
Time (sec)

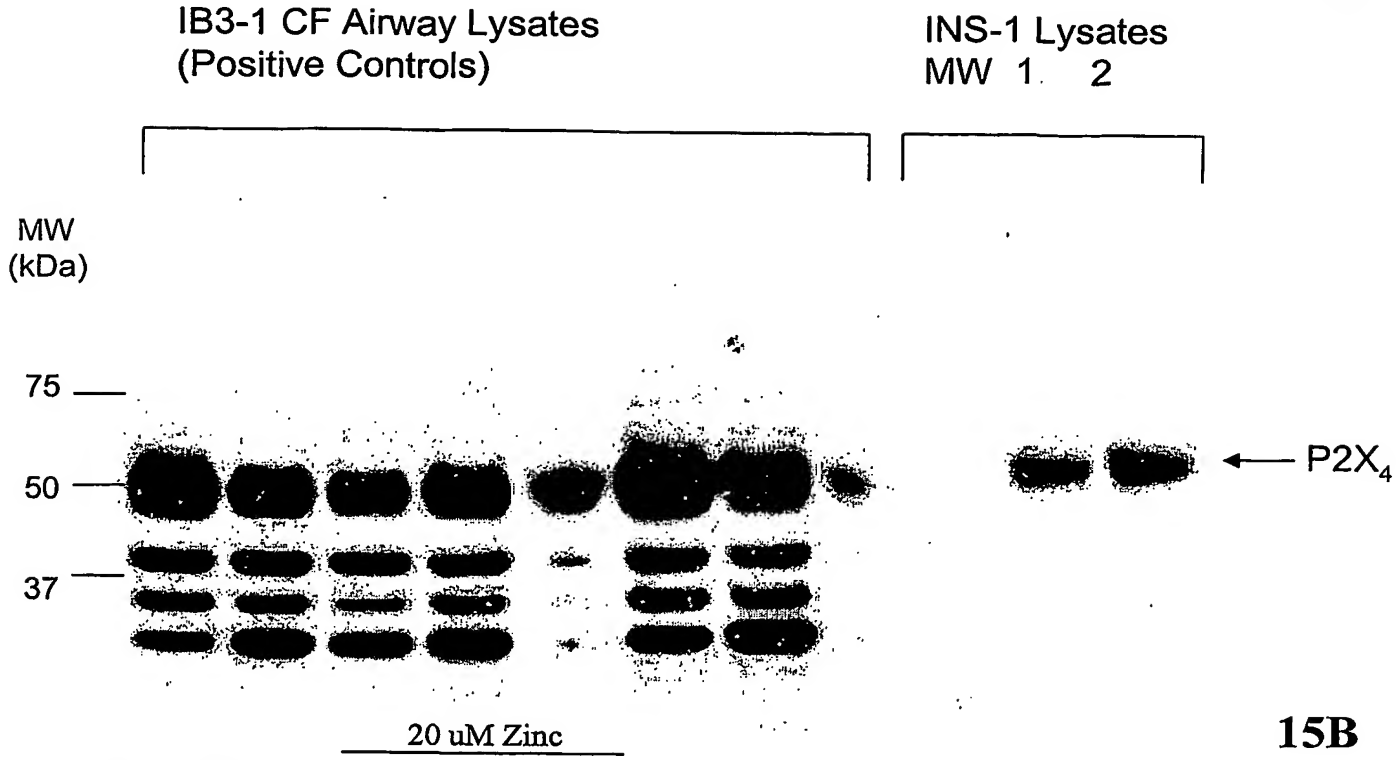
14A


mM Ni2+
0.01
0.1
1
Amil, 10 µM
mM Zn2+
0.01
0.1
1
Amil, 10 µM
5.5
4.2
2.4
4.0
3.5
3.8
2.5

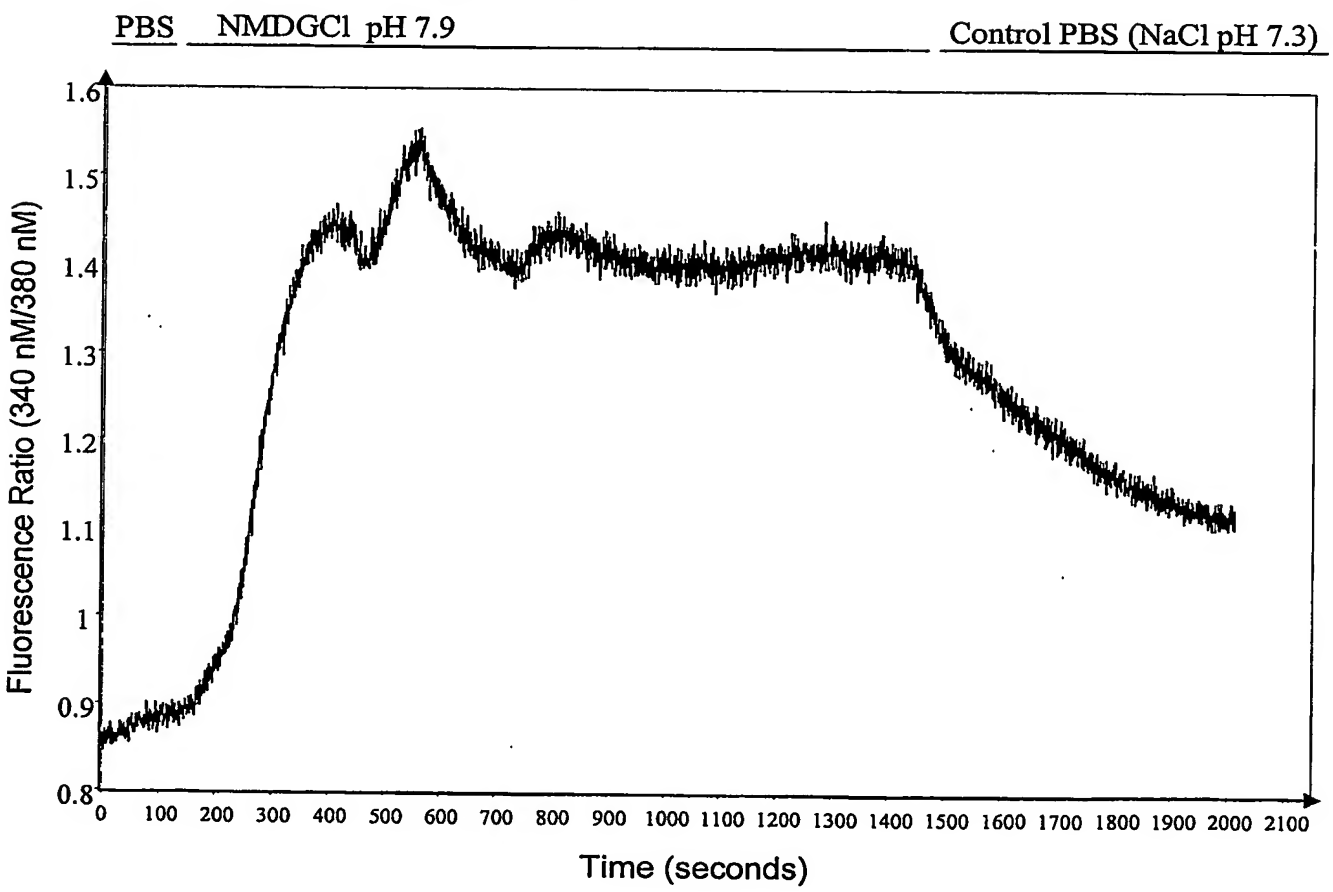
14B


Transepithelial Voltage (mV)
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
-50
Basal
20 µM Phenamil
Basal
10 µg/ml Leupeptin
Zn2+
Basal
20 µM
100 µM
Ni2+
Basal
20 µM
100 µM
Cd2+
Basal
20 µM
100 µM
Mn2+
Basal
20 µM
100 µM
La3+
Basal
20 µM
100 µM

15A


IB3-1 CF Airway Lysates
(Positive Controls)
INS-1 Lysates
MW 1 2
MW
(kDa)
75
50
37
P2X4

15B


20 uM Zinc
PBS
NMDGCl pH 7.9
Control PBS (NaCl pH 7.3)
Fluorescence Ratio (340 nM/380 nM)
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100
Time (seconds)

16A

Modified Saline** (pH 7.3)

Time	Absorbance	[Insulin]
15"	0.682 ± 0.03	~3.0 ng/ml
15'	0.765 ± 0.04	3.25
30'	0.794 ± 0.06	3.5
60'	1.794 ± 0.09	9.0
120'	1.137 ± 0.05	5.0

Modified Saline (pH 7.3) + **15 mM Glucose**

Time	Absorbance	[Insulin]
15"	1.070 ± 0.05	~5.0 ng/ml
15'	0.957 ± 0.07	4.5
30'	1.204 ± 0.10	5.5
60'	2.065 ± 0.05	11.0
120'	1.105 ± 0.18	5.0

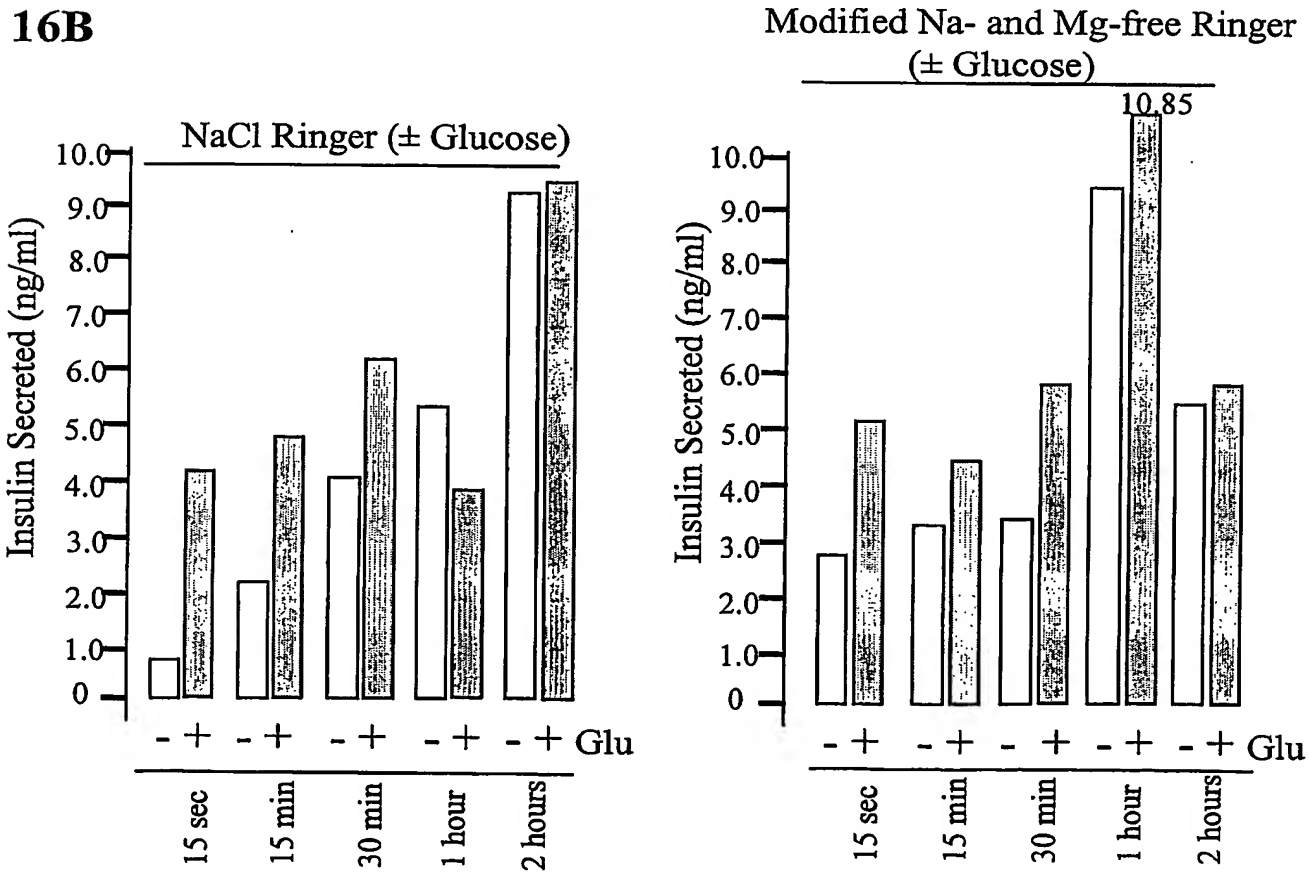
*Generous gift of Dr. Chris Newgard at Duke.

**Modified saline is 0 Na (substituted fully by NMDG), 0 Mg, and 3 mM Ca.

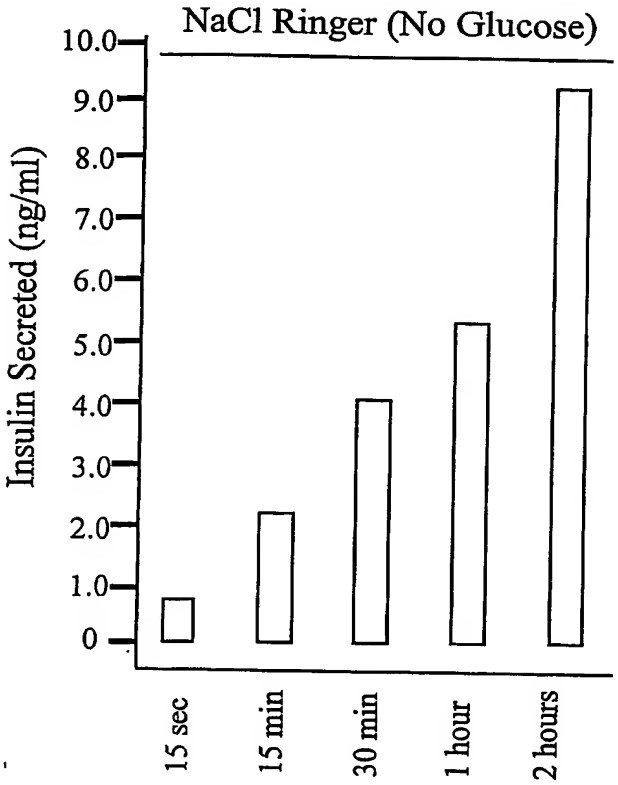
Standard Curve

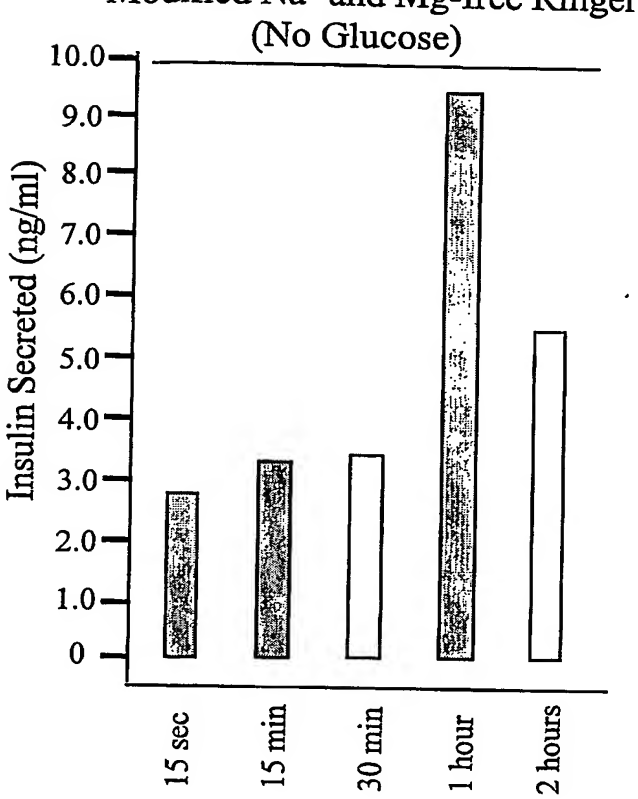
Absorbance	[Insulin]
0.248	0.0
0.226	0.2 ng/ml
0.280	0.5 ng/ml
0.377	1.0 ng/ml
0.559	2.0 ng/ml
1.10	5.0 ng/ml
1.91	10.0 ng/ml
~3.0	~20 ng.ml

16B

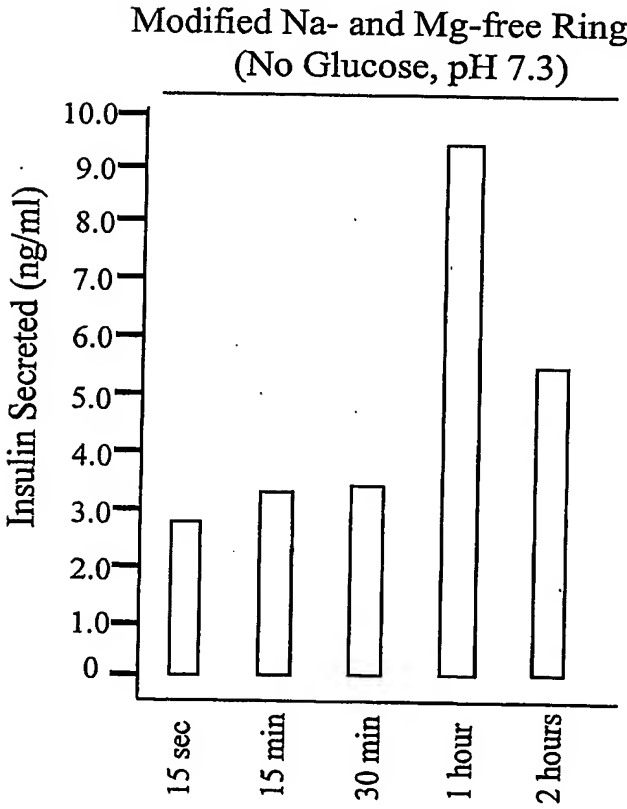


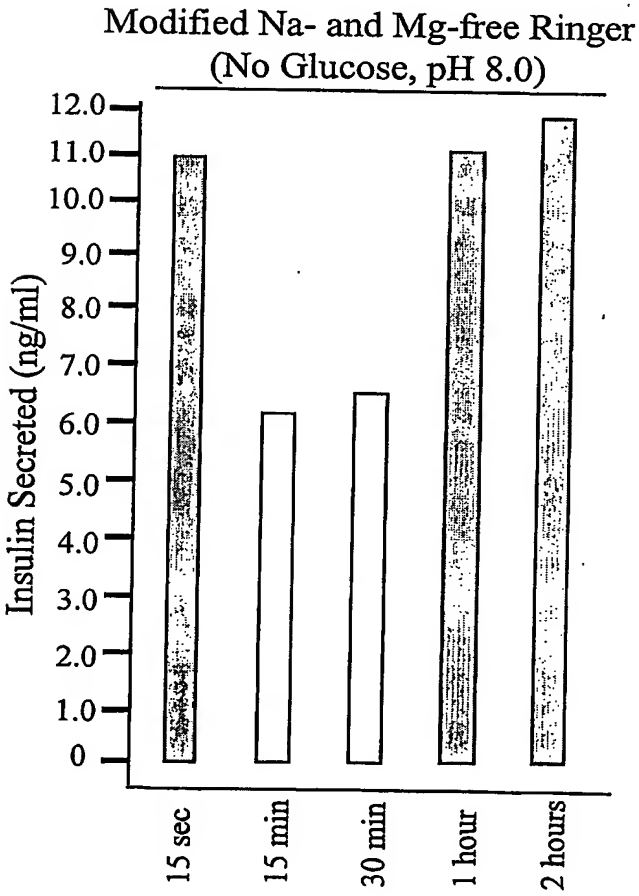
17A

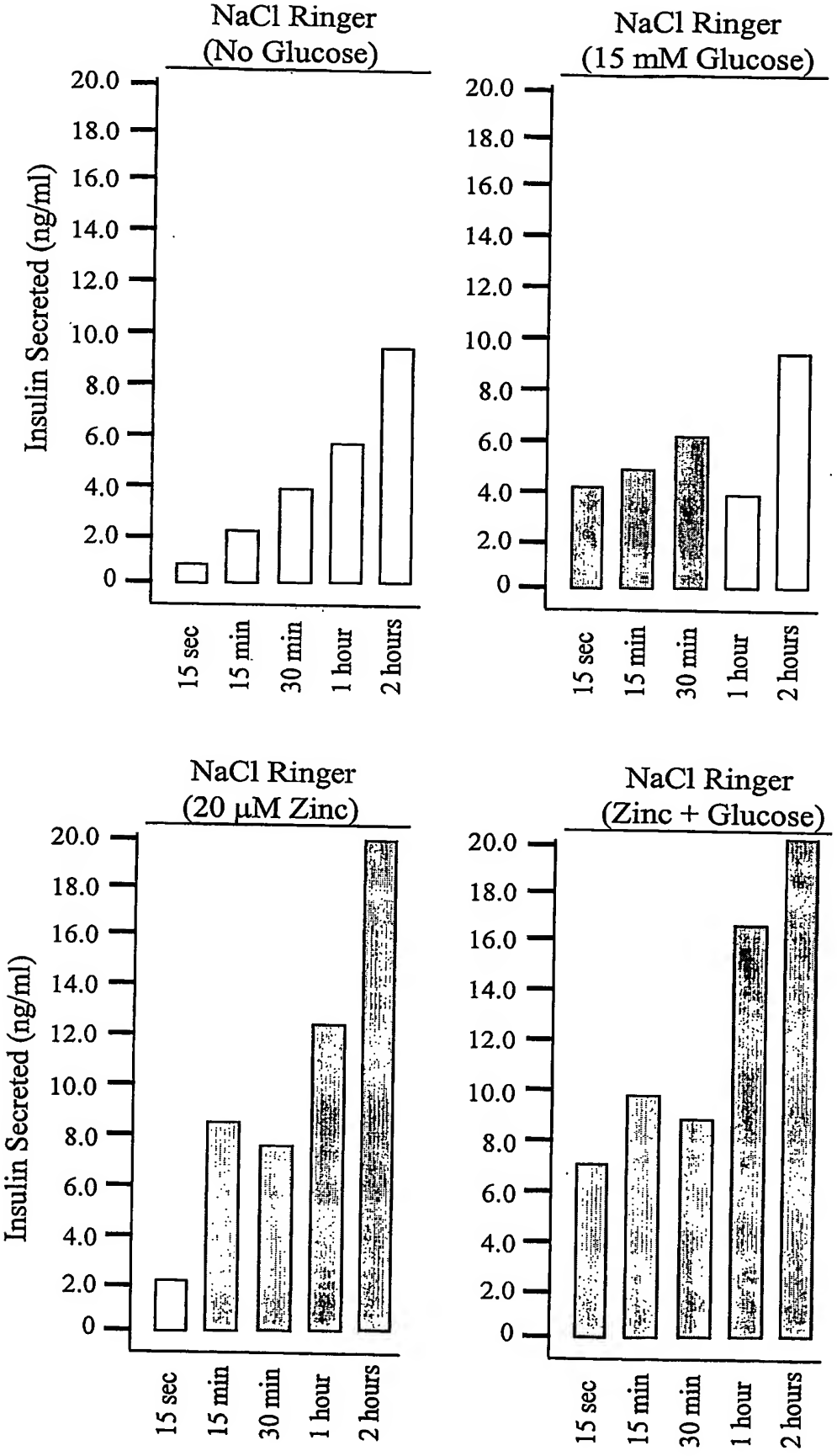

NaCl Ringer (No Glucose)
Insulin Secreted (ng/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours

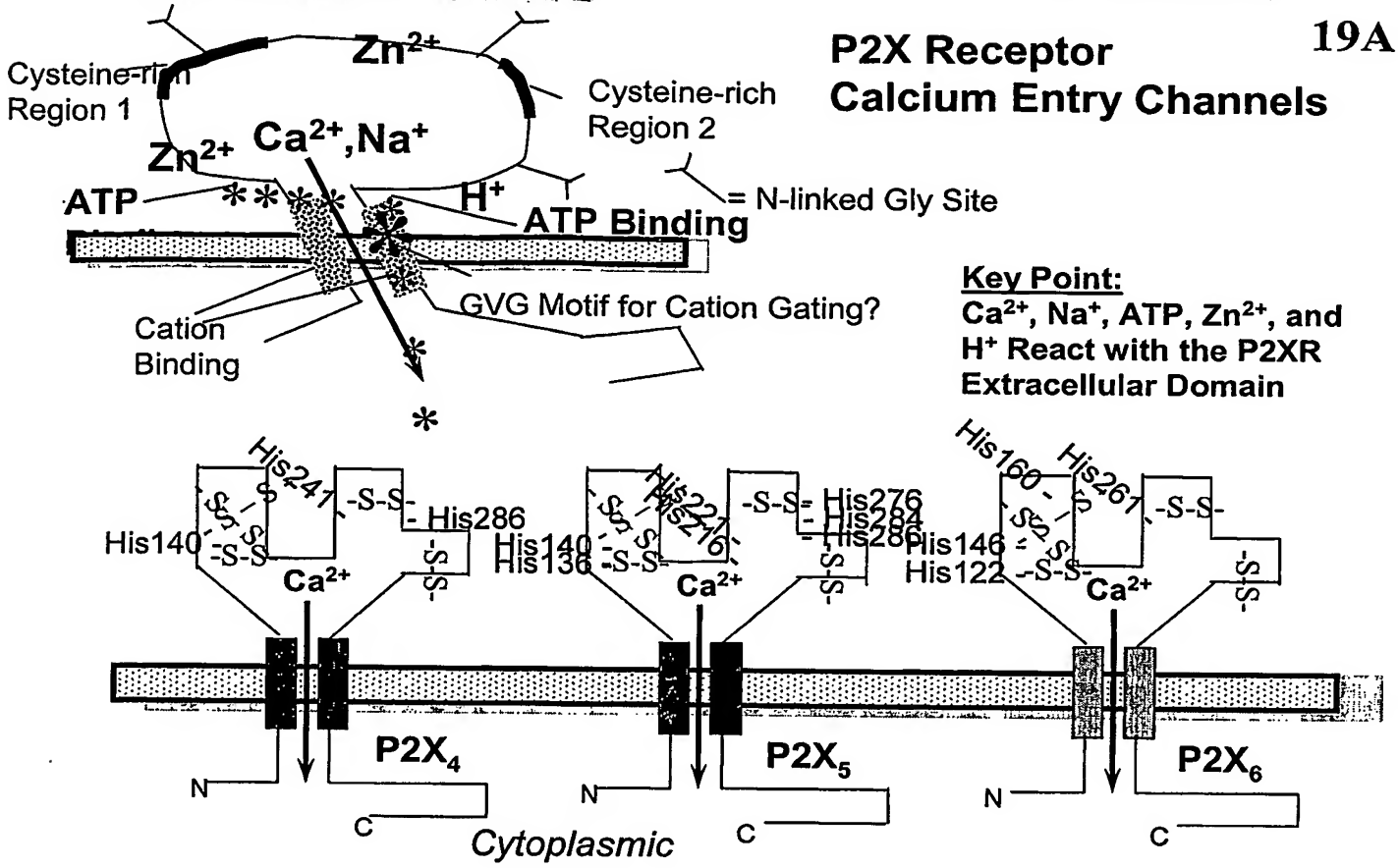

Modified Na- and Mg-free Ringer
(No Glucose)
Insulin Secreted (ng/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours

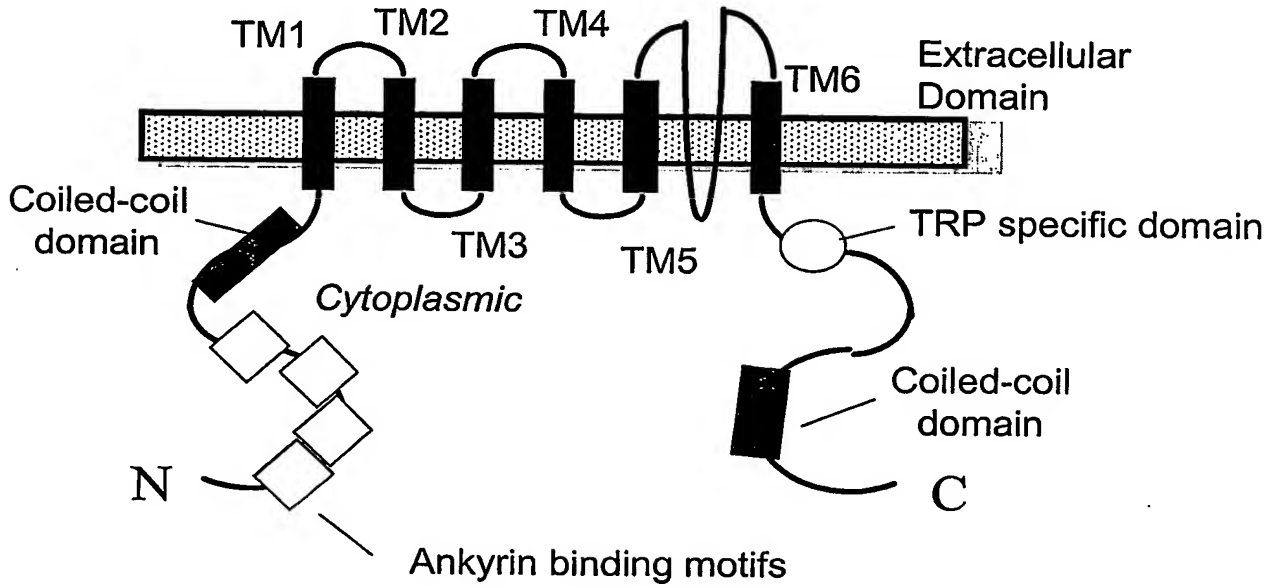
17B

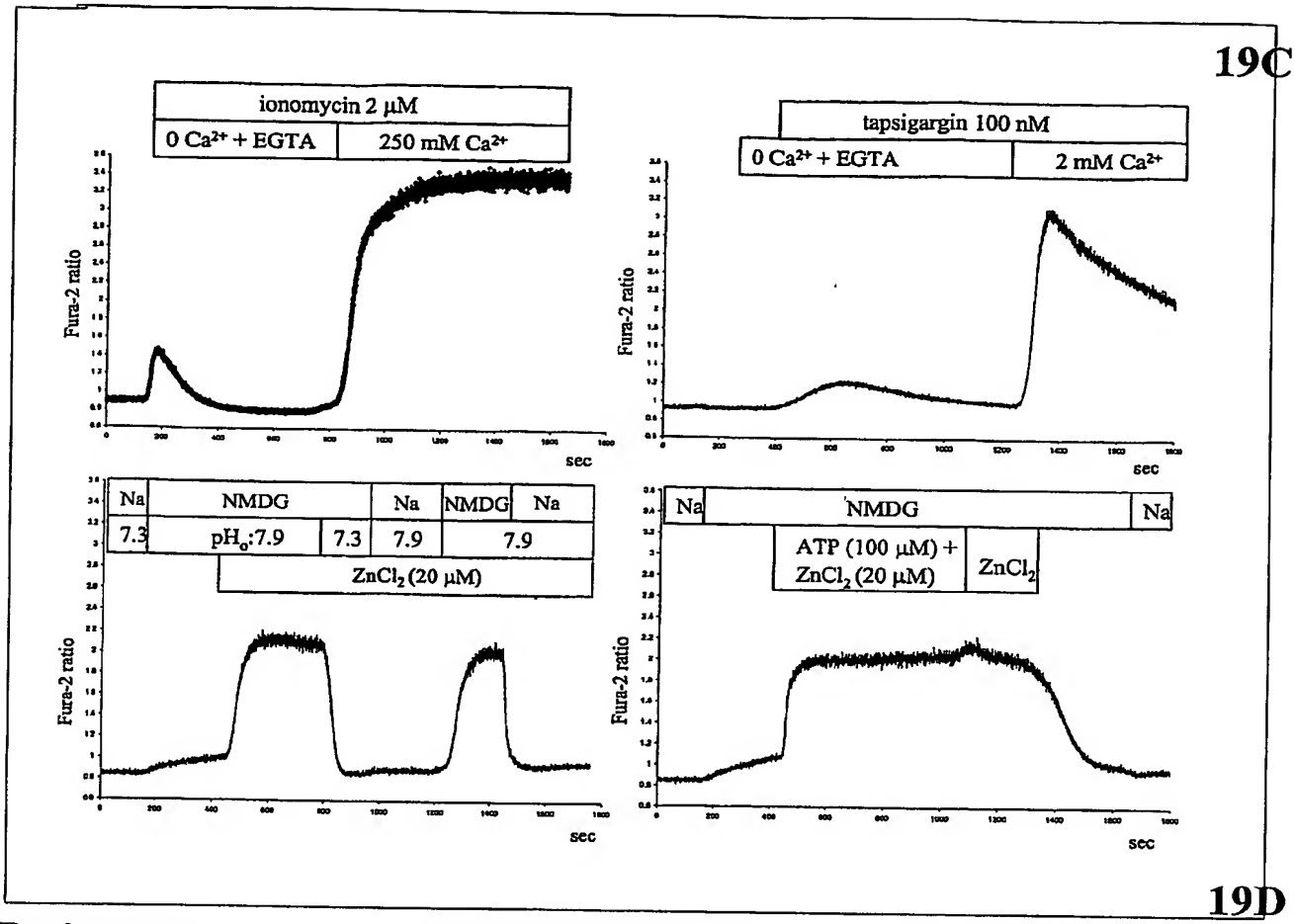

Modified Na- and Mg-free Ringer
(No Glucose, pH 7.3)
Insulin Secreted (ng/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours


Modified Na- and Mg-free Ringer
(No Glucose, pH 8.0)
Insulin Secreted (ng/ml)
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
15 sec
15 min
30 min
1 hour
2 hours


NaCl Ringer
(No Glucose)
NaCl Ringer
(15 mM Glucose)
NaCl Ringer
(20 µM Zinc)
NaCl Ringer
(Zinc + Glucose)
Insulin Secreted (ng/ml)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
15 sec
15 min
30 min
1 hour
2 hours

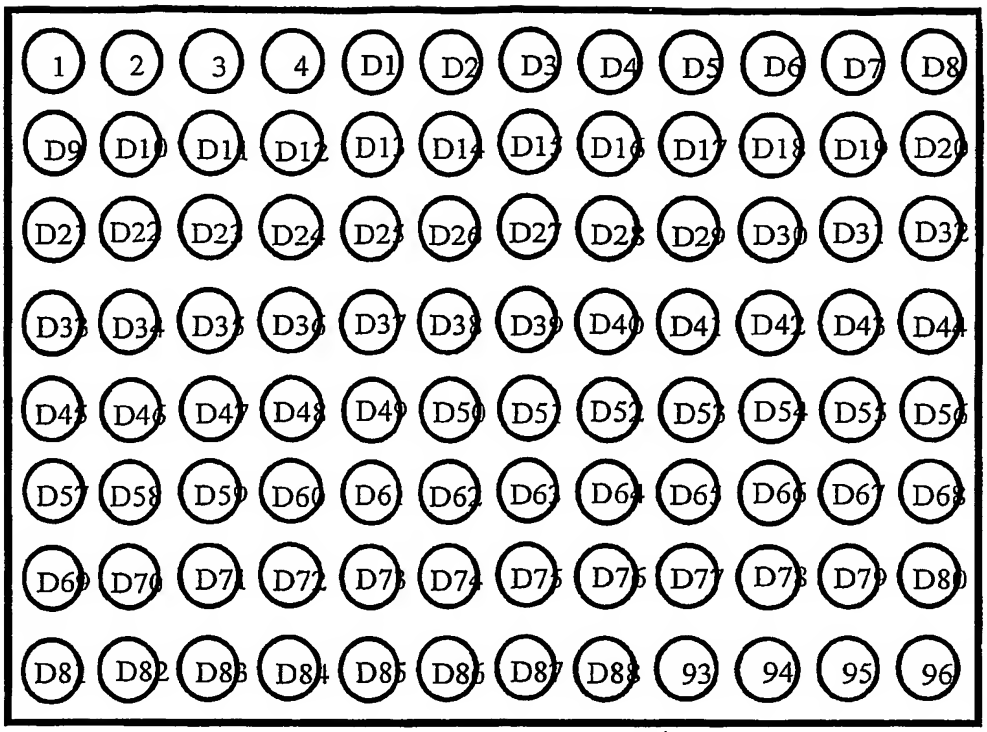

P2X Receptor
Calcium Entry Channels
19A
Cysteine-rich
Region 1
Cysteine-rich
Region 2
Zn2+
Zn2+
Ca2+,Na+
H+
ATP
ATP Binding
= N-linked Gly Site
GVG Motif for Cation Gating?
Cation
Binding
Key Point:
Ca2+, Na+, ATP, Zn2+, and
H+ React with the P2XR
Extracellular Domain
His241
His140
His286
His216
His276
His284
His136
His160
His261
His146
His122
Ca2+
P2X4
P2X5
P2X6
N
C
Cytoplasmic


TRPC Calcium Entry Channels
19B
TM1
TM2
TM3
TM4
TM5
TM6
Extracellular
Domain
Coiled-coil
domain
TRP specific domain
Cytoplasmic
Coiled-coil
domain
N
C
Ankyrin binding motifs

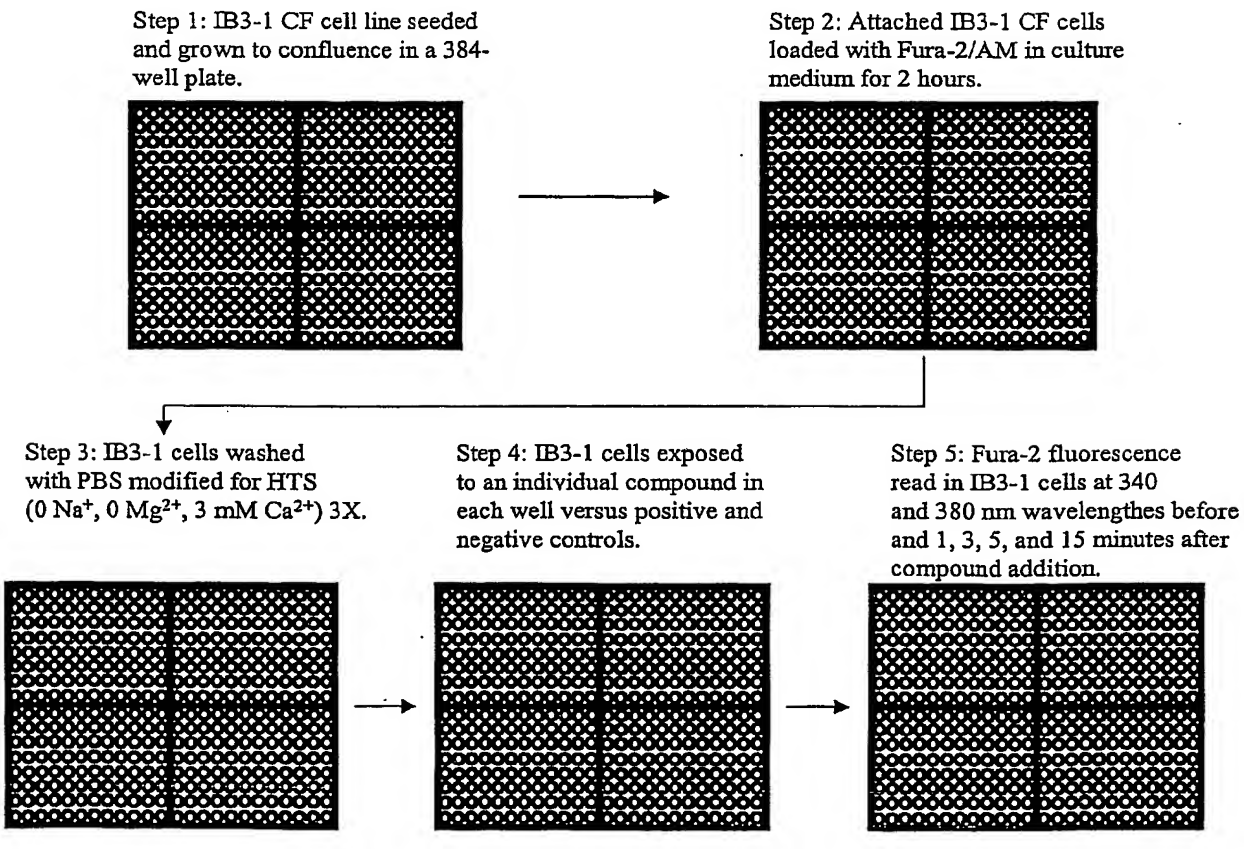


Designation	Mode of Stimulation	Epithelial Polarity
Store-operated Ca^{2+} channels (SOCs) or I_{CRAC}	ER store depletion	Unclear
TRP channels	**ER store depletion (partial) Alkaline extracellular pH (partial)**	**Apical & Basolateral**
P2X receptor Ca^{2+} entry channels	**Extracellular zinc and ATP**	**Apical & Basolateral**
ECaC or CAT *(Related to TRPs)*	**ER store depletion**	**Apical**
Ca^{2+}-permeable non-selective cation channel (NSCC)	Stretch-activated	Apical

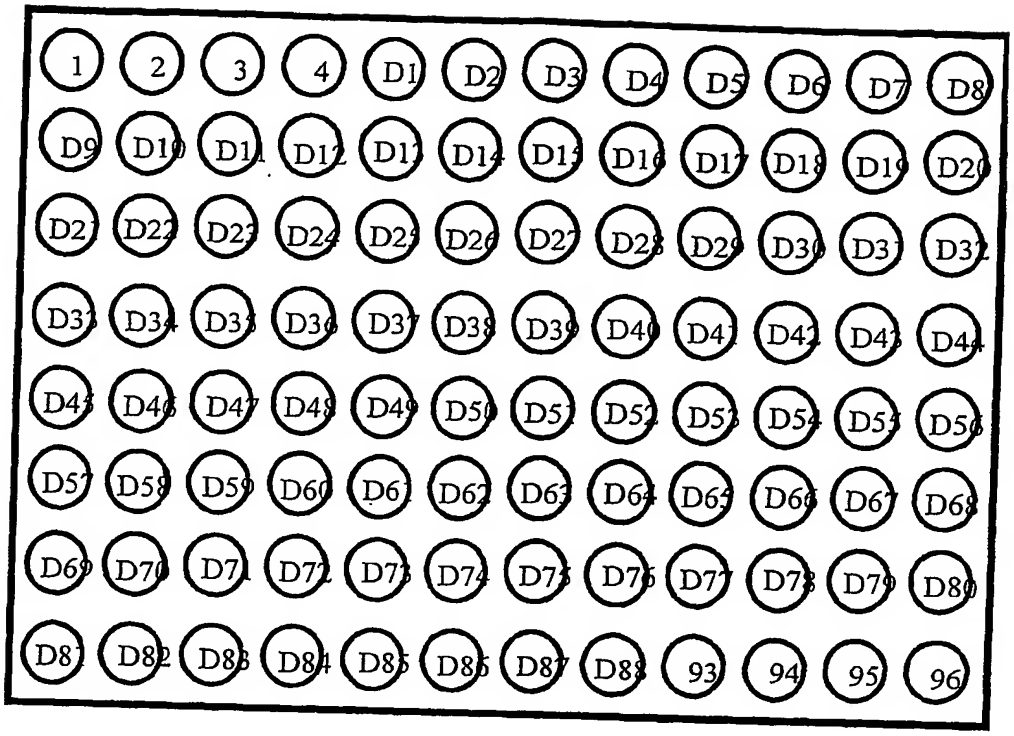
20A


1 2 3 4 D1 D2 D3 D4 D5 D6 D7 D8
D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20
D21 D22 D23 D24 D25 D26 D27 D28 D29 D30 D31 D32
D33 D34 D35 D36 D37 D38 D39 D40 D41 D42 D43 D44
D45 D46 D47 D48 D49 D50 D51 D52 D53 D54 D55 D56
D57 D58 D59 D60 D61 D62 D63 D64 D65 D66 D67 D68
D69 D70 D71 D72 D73 D74 D75 D76 D77 D78 D79 D80
D81 D82 D83 D84 D85 D86 D87 D88 93 94 95 96

20B

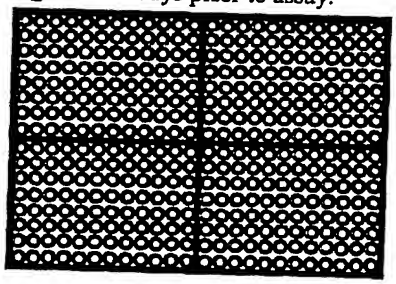

Step 1: IB3-1 CF cell line seeded and grown to confluence in a 384-well plate.
Step 2: Attached IB3-1 CF cells loaded with Fura-2/AM in culture medium for 2 hours.
Step 3: IB3-1 cells washed with PBS modified for HTS (0 Na^+, 0 Mg^{2+}, 3 mM Ca^{2+}) 3X.
Step 4: IB3-1 cells exposed to an individual compound in each well versus positive and negative controls.
Step 5: Fura-2 fluorescence read in IB3-1 cells at 340 and 380 nm wavelengthes before and 1, 3, 5, and 15 minutes after compound addition.

20C

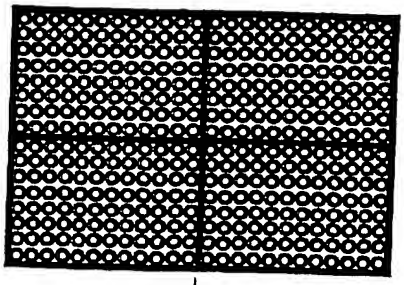


20D

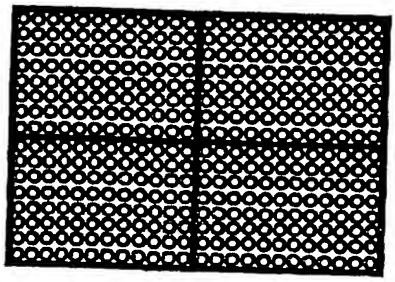
Step 1A: INS-1 β cell line seeded in a 384-well plate.
Step 1B: INS-1 cells rested in 5 mM glucose 2 days prior to assay.



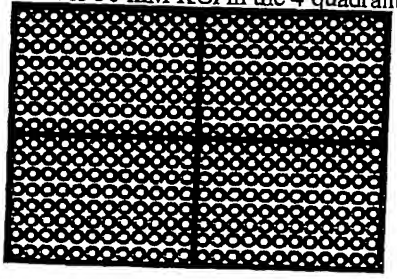
Step 2: Attached INS-1 β cells loaded with Fura-2/AM in low glucose culture medium for 2 hours.



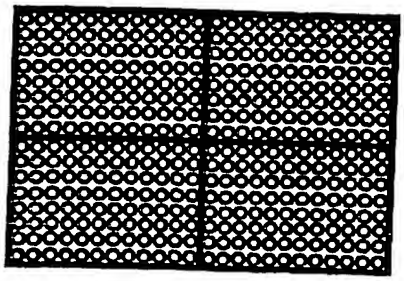
Step 3: INS-1 cells washed with PBS modified for HTS (0 Na^+, 0 Mg^{2+}, 3 mM Ca^{2+}) 3X.



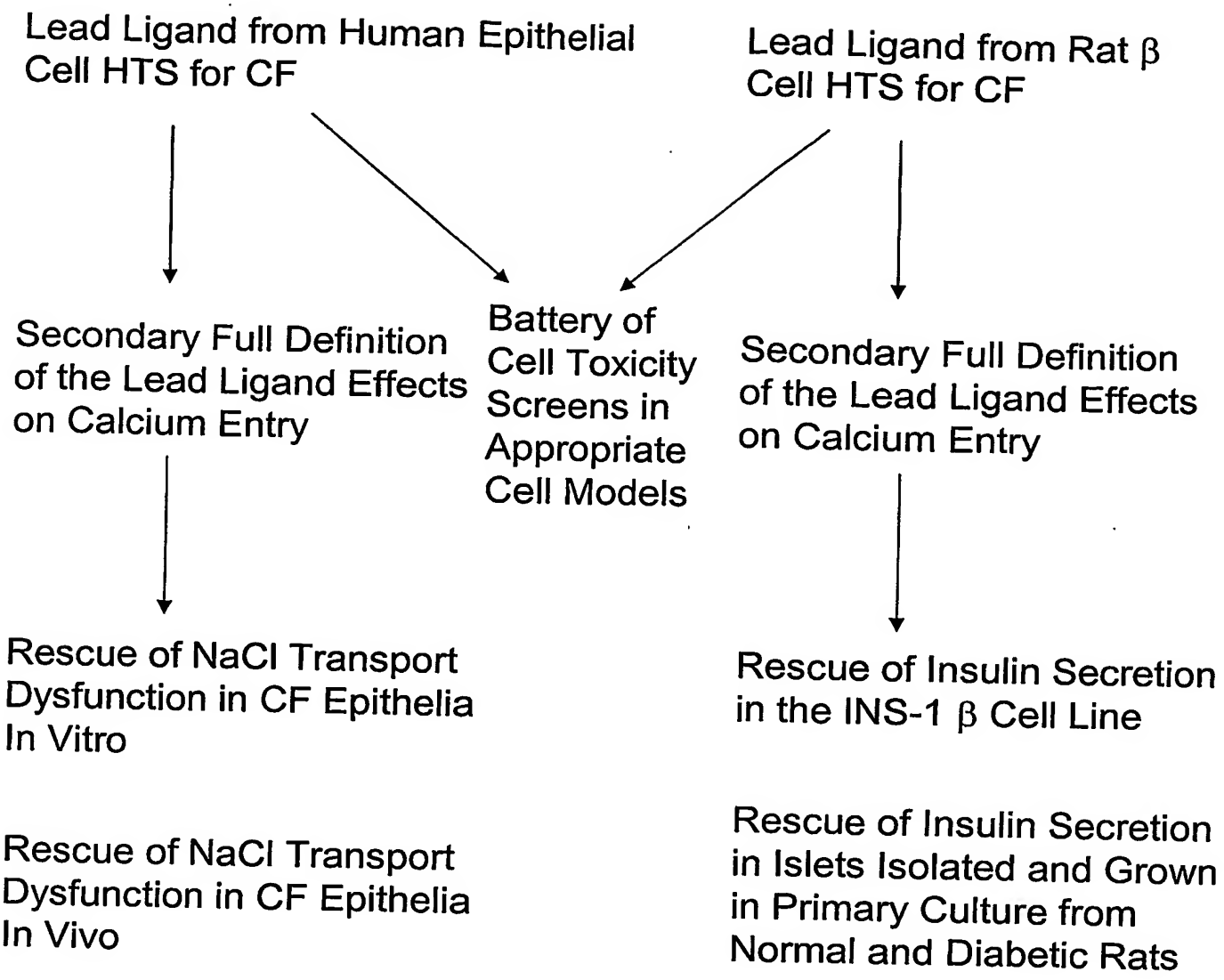
Step 4: INS-1 cells exposed to an individual compound in each well versus positive and negative controls in the absence and presence of 15 mM glucose and/or 30 mM KCl in the 4 quadrants.



Step 5: Fura-2 fluorescence read in INS-1 cells at 340 and 380 nm wavelengths before and 1, 3, 5, and 15 minutes .



20E


Lead Ligand from Human Epithelial Cell HTS for CF
Lead Ligand from Rat β Cell HTS for CF
Secondary Full Definition of the Lead Ligand Effects on Calcium Entry
Battery of Cell Toxicity Screens in Appropriate Cell Models
Secondary Full Definition of the Lead Ligand Effects on Calcium Entry
Rescue of NaCl Transport Dysfunction in CF Epithelia In Vitro
Rescue of NaCl Transport Dysfunction in CF Epithelia In Vivo
Rescue of Insulin Secretion in the INS-1 β Cell Line
Rescue of Insulin Secretion in Islets Isolated and Grown in Primary Culture from Normal and Diabetic Rats